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From the Department for Nervous and Mental Disorders
on "Filadelfia", Dianalund, Denmark.

The Excitability of the Respiratory Centre during Sleep and during Evipan Anaesthesia.

By

TORSTEN ØSTERGAARD.

Received 31 March 1944.

It is generally recognized that carbon dioxide is the adequate stimulus for the respiratory centre, so that alterations in the CO_2 tension of the blood will produce corresponding alterations in the pulmonary ventilation (HALDANE and PRIESTLEY 1905, M. NIELSEN 1936). The amount of ventilation does not depend solely on the strength of stimulus, the excitability of the centre being an important variable factor (COHNSTEIN and ZUNTZ 1888).

The excitability is estimated in human beings according to LINDHARD (1911) by exposing the subject to known CO_2 tensions and measuring the resulting ventilation. An increase in excitability was found after suitable doses of strychnine and also at low oxygen pressure, while morphia, chloral and increased oxygen pressure reduced the excitability. At transition from rest to muscular work KROGH and LINDHARD (1913) pointed out an increase in excitability, and finally it may be mentioned, that in certain cases of melancholia a decrease in excitability was observed by SCHOU, TROLLE and ØSTERGAARD (1942).

In the present paper the changes in excitability due to sleep and evipan narcosis are studied.

Sleep.

It is generally assumed that sleep reduces the excitability of the respiratory centre and this is concluded either from deter-

minations of alveolar CO_2 tension which was found increased (STRAUB 1915, BASS and HERR 1922, ENDRES 1923, RABINOWITSCH 1929) or from a reduced ventilation (GUYER 1928, FLEISCH 1929), but direct determinations of the excitability are lacking except in an unpublished paper by MAGNUSSEN, which the author has had an opportunity to consult.

The method employed is that of LINDHARD (1933), who has modified the method given by COHNSTEIN and ZUNTZ (1888). The subject breathes different mixtures of air and CO_2, and corresponding values for the alveolar CO_2 tension and the ventilation are determined. These are plotted with the alveolar CO_2 tensions as abscissa and the ventilation as ordinate. The resulting curve is normally a straight line and its point of intersection with the abscissa, corresponding to zero ventilation, is called the point of apnea. NIELSEN showed (1936) that ventilation is in fact suspended just at this point. LINDHARD considered the CO_2 increases above this point as "effective stimuli" and constructed curves relating the increases in ventilation obtained by breathing CO_2 mixtures to the effective stimuli. After this transformation the steepness of the curve found gives a measure of the excitability which can be expressed, when all curves are drawn to the same scale, either by the angle itself or by a function like the tangens. The latter is chosen in the present paper.

During the experiment the subject rests on a bed and must have been at rest for at least half an hour before any determination is made. He respires through a mouthpiece and valve, and the nose is closed by a clip (KROGH 1923). He inspires either directly from the atmosphere or from a cylinder containing a suitable CO_2 mixture in air and with a Douglas bag interposed. The expired air is collected in another Douglas bag and afterwards measured by emptying the bag through a gas-meter. The alveolar CO_2 tension is obtained by analysis in a HALDANE-KROGH apparatus of a sample collected during a number of inspirations (15—20) from the expiration tube close to the valve (KROGH and LINDHARD 1914). The analytical results, accurate to 0.02 %, are converted into mm CO_2 tension.

The experiments on spontaneous sleep were made in the evening at normal bed time of the subject. They will succeed only on "good sleepers" even when conditions are improved by reducing the amount of sleep for 1—2 nights previously. Even so the subject will wake up after $^1/_2$—$1^1/_2$ hours on account of the inconvenience unavoidably connected with the experimental conditions.

As breathing of the CO_2 mixture can by itself disturb the sleep it was found convenient to initiate sleep during a period of CO_2 breathing and to change later to amospheric air. This was done in all experiments

except the very first. Only one period with CO_2 breathing could be obtained, and a concentration of 4—6 % in the inspired air was chosen, as higher ones were apt to disturb sleep and lower ones produced only a very slight increase of ventilation.

The state of sleep was ascertained by asking the subject first in a whisper and then in a half whisper: "Do you sleep?" The subject was instructed to react by opening the eyes and when this reaction failed was assumed to be asleep. The results show that this simple expedient was sufficient.

During sleep and especially during anaesthesia a leak may occur at the mouthpiece because the perioral muscles will relax and it is necessary to watch closely for this and eventually to support the chin by a bandage.

The two subjects in spontaneous sleep were falling asleep very easily. The last one has been used for all experiments with evipan sleep and for one single anaesthesia experiment.

Results.

Figure 1 shows an experiment on spontaneous sleep. The subject was very sleepy, and went immediately into sleep as the permission was given. Then there was a restful and uninterrupted sleep for 80 minutes. Ventilation, alveolar CO_2 tension, frequency and depth of respiration are recorded, partly during atmospheric breathing and partly during breathing of air with $4^1/_2$ % CO_2. The sleep causes a fall in ventilation, amounting to 20 % for the CO_2 breathing and likewise to about 20 % (17—23) for the atmospheric breathing. To the right on the figure two excitability curves for a waking state and for sleep are shown. A pronounced displacement in a depressive direction is seen during the sleep. The excitability of the respiratory centre has thus been reduced. There is a displacement of the point of apnea, from 29 mm tension in waking state to 36 mm tension in sleep. LINDHARD's steepness curves are constructed and the tangents of the steepness angles are calculated at 1.41 in waking state and at 1.10 in sleep for this subject (when the unity 1 liter on the ordinate scale corresponds to 20 % on the abscissa).

Figure 2 shows likewise an experiment with spontaneous sleep, but here the subject went into sleep during the CO_2 breathing. After 20 minutes CO_2 breathing, at *Y*, the subject is permitted to sleep and dozes off immediately. The ventilation begins to fall, and is further reduced when sleep sets in. The alveolar carbon dioxide tension rises 4 mm. The ventilation falls during the fol-

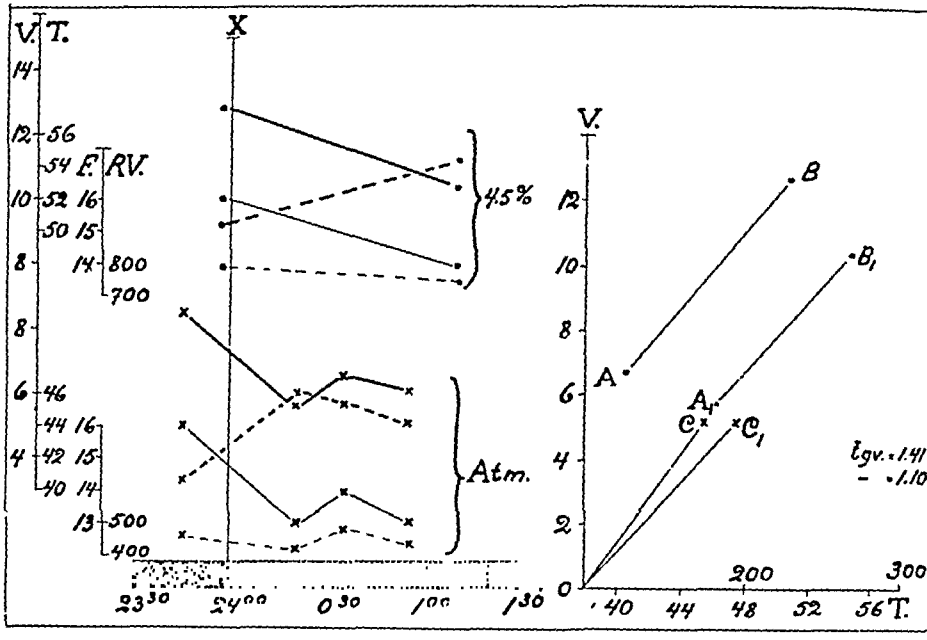
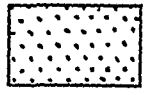
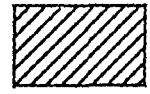


Fig. 1. Experiment with spontaneous sleep (♂ P., 29 years).

V.: Ventilation in liters per minute (————).
T: Alveolar CO_2 tension in mm Hg. (— — — — —).
F.: Frequency per minute (————).
RV.: Depth of respiration in ccm (— — — — —).
AB and OC: Curves of excitability for a waking state.
A_1B_1 and OC_1 the corresponding curves for sleep.

Sleepiness:  Sleep: 

lowing atmospheric breathing under sleep to a value below the initial waking value. — The depressive displacement of excitability during sleep is not quite so pronounced as in the first experiment. This appears from the curves, but especially from the difference between the tangent of the two steepness angles having the values of 1.33 for a waking state and 1.13 for sleep. The difference may be due either to individual variation or to the sleep being more superficial. From the actual experimental record one gets the impression, that the latter is the case; a later experiment on the same person with sleep after giving of evipan seems to confirm this supposition. — Characteristically, in this experiment there is no displacement of the apnea point, which has the tension 30 mm in waking state and in sleep.

In the following experiments the sleep was induced by evipan in hypnotic doses, given by intramuscular injection of 10 %

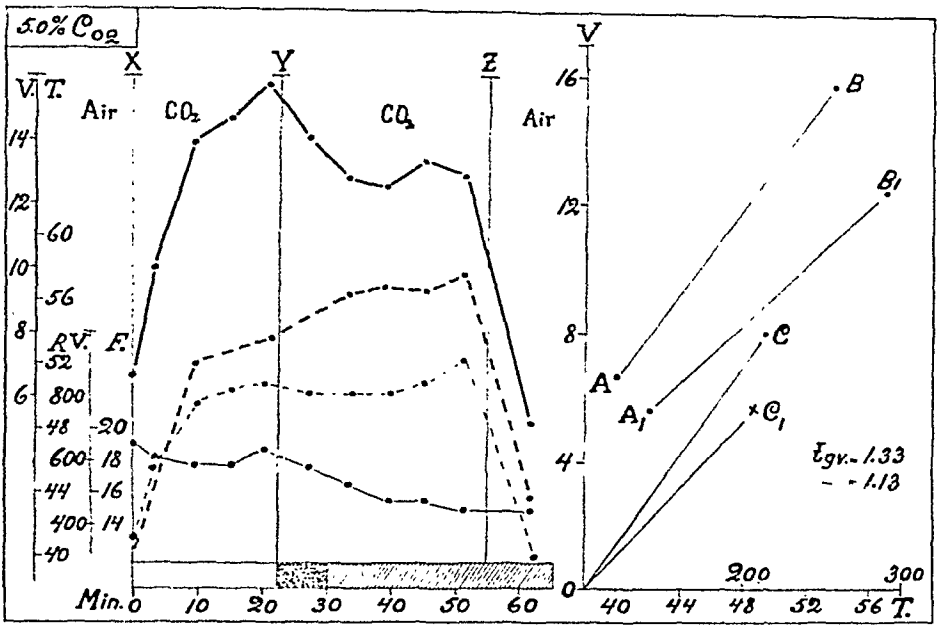


Fig. 2. Experiment with spontaneous sleep (♀ S., 42 years).
Legends: As in fig. 1.

freshly prepared solution. First two introductory experiments may be mentioned with sleep after $^1/_2$ and 1 gram of evipan respectively. In both cases 5.6 % CO_2 was during the whole time inhaled in atmospheric air, and at the point when the ventilation had reached maximum the evipan was injected. A few minutes later drowsiness occurred, and after 10 and 14 minutes sleep set in. The fall in ventilation (20 and 34 %) was largest with the larger dose of evipan, exclusively on account of a greater depth of sleep, as seen later on. In the first experiment a period of sleep of 40 minutes was recorded, during which both ventilation and tension showed rather constant values. In both cases the frequency decreased (by 2—4 per minute). In one of the experiments the subject was awakened from a deep and restful sleep and asked to keep awake for a time. It was seen, that the ventilation during this period rose to a little above the waking value before the giving of evipan, while the alveolar CO_2 tension fell to a little below the previous level. During the ensuing sleep values like those of the first period of sleep were recorded. This indicates, that it is exclusively the state of sleep and not the hypnotic given in itself, which has a depressive effect on the excitability of the respiratory centre.

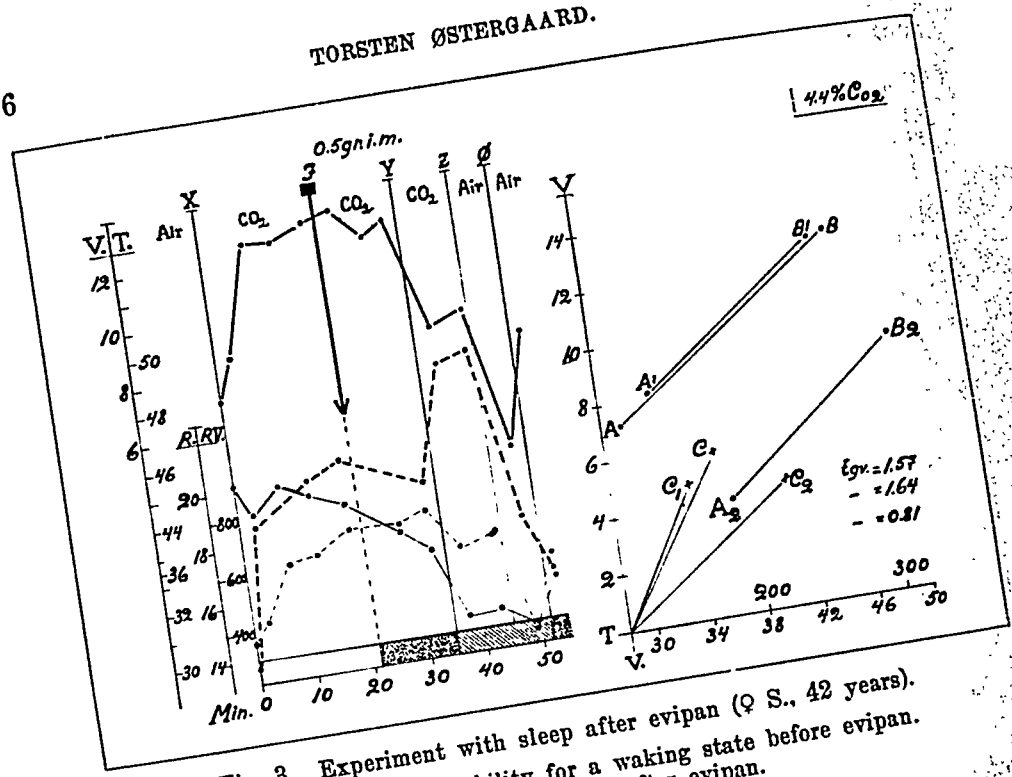


Fig. 3. Experiment with sleep after evipan (♀ S., 42 years).

AB and OC curves of excitability for a waking state before evipan.
A_1B_1 and OC_1 curves for a waking state after evipan.
A_2B_2 and OC_2 curves for sleep after evipan.
At *1.*: Injection of evipan solution (10 %) intramuscularly.

In the experiment *figure* 3 investigation is made, whether this supposition holds good. After the evipan was given the subject is asked to keep awake and exerts herself to do so. The eyes are widened spasmodically; she gets simple calculations to do and is to state the results by means of her fingers. It is seen, that the ventilation during this period does not show any real fall, but fluctuates round the value of the time immediately before the evipan injection. The alveolar tension does not rise, but shows a slight fall. At the time of Y the subject is allowed to sleep and falls into sleep almost immediately. At once a considerable fall in ventilation amounting to 34 % appears, and an abrupt increase in the tension of 3.7 mm. At Z, while the sleep continues, atmospheric air is again breathed and ventilation is here 38 % lower, the tension 6 mm higher than in the waking state. After Ø the subject is kept awake, and we have a waking state after giving of evipan during atmospheric breathing. — The frequency also here shows a pronounced fall during the sleep (19—14½), while the depth of respiration does not change characteristically. — In comparing the values for ventilation and alveolar tension

in the three phases, three curves of excitability are obtained, for a waking state without any hypnotic, for a waking state after giving of evipan and for sleep with evipan. It appears that the curves for the two first states practically coincide, which proves that the evipan in hypnotic doses does not affect the respiratory centre. The excitability is not reduced until sleep sets in. — This has been confirmed by three other experiments during sleep on the same subject, not recorded here. — The excitability curve for sleep in this case is displaced considerably more in a depressive direction than seen in the experiments during spontaneous sleep. As this is not due to any effect of evipan, the cause must be searched for in a deeper sleep under the influence of the hypnotic. The conclusion may be drawn, that the reduction in the excitability of the respiratory centre during sleep increase proportionally with the depth of the sleep. — The tangent of the three steepness angles is here 1.57 for waking state, 1.64 for waking state after evipan and 0.81 for sleep. — The point of apnea is at the tension of 10 mm in waking state and at 26 mm in sleep, which also indicates a considerably reduced excitability in this case. — It is remarkable that the point of apnea in a waking state in this case differs very much from the point obtained in another experiment on the same person (figure 2). Surely the reason is that this experiment is performed in the morning, the former in the evening.

Anaesthesia.

We have seen, hat evipan in hypnotic doses did not influence the excitability of the respiratory centre. In the following the effect of evipan in anaesthetic doses is examined.

The evipan was first taken into use by WEESE (1932), who found that it was an excellent anaesthetic, quickly paralysing the sensory and reflexogene central organs, while the affinity to the centres vital to life, thus the vasomotor and respiratory centres, was found to be extremely slight. WEESE, however, by his investigations on cats (1933) saw that death by giving of lethal doses always occurred by paralysis of the respiration.

Still he emphasizes (1935), that "during an ideal anaesthesia with evipan the effect on the respiration and circulation is strikingly slight". — Several authors even state, that respiration is unaffected, and so the Danish treatise on pharmacology (MØLLER 1941 and 1943). Nu-

merous authors report, that the respiration is often superficial to the point of being quite inaudible and that there may be slight cyanosis — consequently an indication of a certain effect (SØNDERGAARD 1942, MADSEN and SCHNOHR 1942, and many others).

As a matter of fact it is in the field of respiration, that the most grave complications have occurred, temporary or dangerous cessations of the respiration, and in several cases death by paralysis of the respiration in consequence of overdosage (VOIGT 1936, WESTERBORN 1937, and others). WESTERBORN states, that "the typical evipan death is paralysis of the respiration directly connected with the anaesthesia".

During these investigations I have attempted to elucidate, to what a degree the respiration is affected during the ordinary evipan anaesthesia without any complications. — Such investigations have not been made previously on human beings.

On apes STORM (1935) examined the respiratory movements of the thorax and the blood pressure during evipan anaesthesia. In good anaesthesias no influence was observed. By a quick injection of large doses the respiration was always paralysed before the circulatory system. After a certain time, possibly after artificial respiration the respiration and the circulation sets in again, the circulation first. — DONATELLI and INGIALLE (1940) caused in rabbits paralysis of the respiration by giving large doses of evipan and showed that the animals might be kept alive by means of artificial breathing, until the centre in the course of some minutes was de-poisoned through the decomposition of the evipan and the spontaneous respiration again set in. Consequently there was no paralysis of other vital centres. — BEECHER and MOYER (1941) observed in dogs at an early stage of evipan anaesthesia a considerably reduced reaction to an increase of the carbon dioxide content of the inspired air, indeed, large doses of CO_2 even increased the depressive effect of the evipan on the centre.

I have examined 8 cases of anaesthesia in 4 persons. They were all of short duration, from 6 to 18 minutes. Only one subject was examined in a state of deep anaesthesia, in the others the anaesthesia was light, as seen from the fact, that the reaction to pain by pin-pricks was only suspended for a few minutes.

Two female patients — one cured of melancholia, the other a psycho-infantible, but otherwise psychically and somatically sound — voluntarily and readily gave their permission to the experiments. The third subject was an 18 years old, slightly inferior male patient, who received 4 evipan anaesthesias for a suggestive purpose. The fourth was a female patient with a chronic catatoniform schizophrenia. No one complained of inconvenience after the anaesthesia - - all of them were able to walk home ½—1 hour after the experiment.

The injection of the freshly prepared solution (10 %) was administered according to the individual requirement, constantly with due regard to momentary state — at first somewhat quicker injection, 1—2 ccm per minute until sleep, then more slowly with $^1/_2$—1 ccm per minute. During the whole time cornea reflex conditions and sensibility to pin-pricks were registered. — 4.3 to 7.5 % CO_2 has been mixed in the inspiratory air. — The mouthpiece has been closely watched with a view to possible leakage.

Results.

In the first experiment a very short anaesthesia was obtained, lasting 6 minutes with $^1/_2$ gram of evipan injected in $2^1/_2$ minutes. In spite of the slight anaesthesia a considerable fall in ventilation appeared (48 %) and a rise in tension. The anesthesia was followed by a restful sleep, from which the subject was awakened and kept awake for 5 minutes, during this period the ventilation and tension adapted themselves to waking values to return to sleep values during the following sleep. — In the second experiment an anaesthesia lasting 13 minutes was caused by 14 ccm evipan solution (1.4 gram of evipan). The ventilation in this case decreased by 78 %. The alveolar CO_2 rose correspondingly considerably, 8.2 mm. This effect on the respiration surprises because the anaesthesia was not deep, reaction to pain only suspended for a few minutes. Frequency and depth both decreased, from 24 to 20 per minute and from 1,400 to 400 ccm. — The anaesthesia continued into a restful sleep during a quarter of an hour, after which the subject awoke spontaneously.

It would require a very protracted anaesthesia to obtain both an atmospheric and a CO_2 breathing with the necessary introductory periods within the case. Therefore I have chosen to divide the experiment and institute the carbon dioxide breathing on one day and the atmospheric breathing on the following day. Certain sources of error are hereby introduced: the anaesthesias cannot be exactly of the same depth and the corresponding values are not registered in exactly uniform phases. But the results at least give an approximate expression of the conditions.

Figures 4 and *5* show the results of two double experiments executed in the manner stated. The first subject, a young man, was — as most young people — resistant against evipan in a pronounced degree; the two anaesthesias were shortlived and not very deep in spite of rather large doses of evipan. The reaction

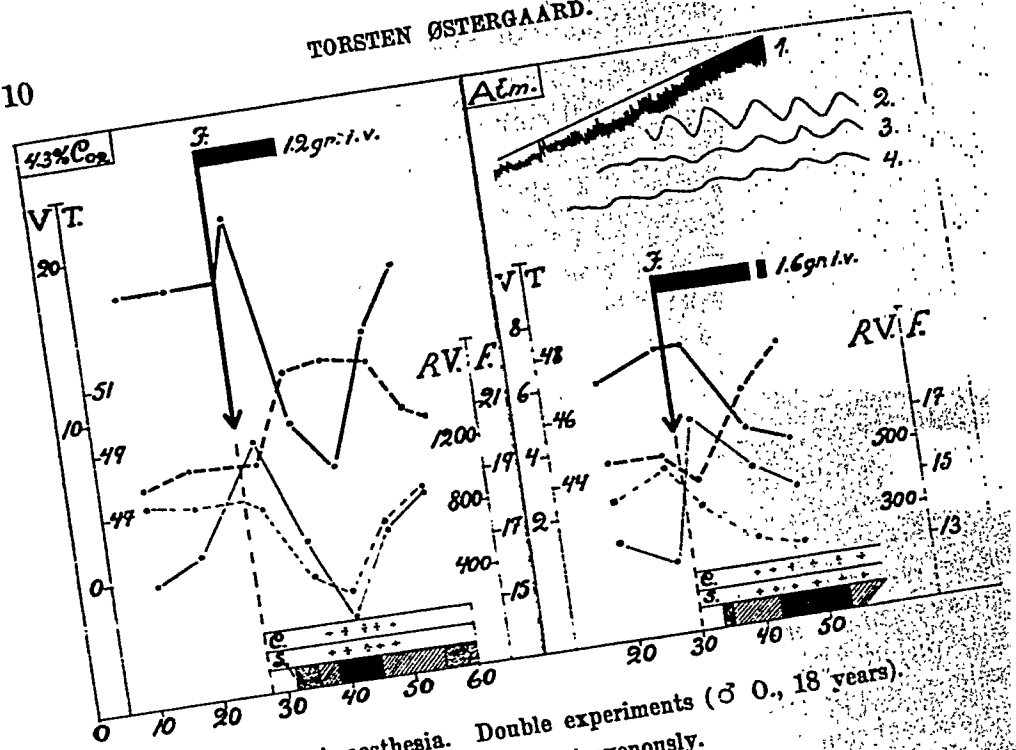


Fig. 4. Anaesthesia. Double experiments (♂ O., 18 years).

At *I.*: Injection of evipan solution intravenously.
c.: Cornea reflex condition.
s.: Reaction to pain by pin-pricking.
Abscissa: Time in minutes.
On the figure spirograms at the top, taken before and during anaesthesia.
(1): Curve recorded on slowly moving drum, transition from sleep to anaesthesia.
(2): Quickly moving drum, waking state. (3): Transition from sleep to anaesthesia.
(4): Anaesthesia.

Anaesthesia: [redacted]

to pain was only suspended for a couple of minutes at the deepest point of anaesthesia. A little nervousness was present, which expressed itself in a slight hyperventilation immediately after beginning the injection. The ventilation in anaesthesia falls 67 % (CO_2 breathing) and 49 % (atmospheric breathing). The tension rose both days 3 mm, a rather slight increase.

The increase in tension does not always correspond in degree to the decrease in ventilation during anaesthesia, but this is probably due to a systematic error, as the respiration is often so superficial, that one cannot be sure of obtaining alveolar samples on account of a deficient washing out the dead space.

The latter experiment had to be interrupted, just as the anaesthesia had occurred, as the respiration suddenly became troubled. There was no paralysis of the respiration, the movements of the thorax

continued regularly, but seemed to be mechanically hindered, and no air was expired into the Douglas bag. Relieved of mouthpiece and noseclip and placed in a side-position the subject again respired unhindered.

In this case the anaesthesia was not deep enough to suspend the reaction to pain.

On this subject respiration curves were recorded by means of KROGH's metabolism apparatus and selected parts are shown in *fig. 4.*

The excitability curves for the experiment just described are shown in *figure 5*, AB and A_1B_1. The apnea point in waking state has the tension 42.4 mm, in anaesthesia 41.6 mm. A displacement in contrary direction of the one taking place in sleep is seen here. — The decrease of excitability during anaesthesia is considerable. Expressed by tangent to the steepness angles the excitability coefficient for waking state is 1.38 and for anaesthesia 0.67. The respiratory centre is much more affected during anaesthesia than during sleep.

In a further double experiment on another subject comparatively deep and protracted anaesthesias of 14 and 18 minutes were obtained by 1.5 gram evipan. The reaction to pain was suspended for 10—15 minutes. The fall in vetilation amounted to 80 % during CO_2 breathing and to 49 % during atmospheric breathing. The tension rose considerably, 8.3 and 8.0 mm respectively on the two days. Both frequency and depth decreased, 18—13 and 1,200—300 ccm. — Curves of excitability are shown in *figure 5*, MN and M_1N_1. It is more pronounced here than in the previous case, that the excitability against large doses of carbon dioxide is proportionally more reduced than against small ones. For the same reason the fall in ventilation expressed in percent is greater in the CO_2 breathing experiments than in the atmospheric experiments. The point of apnea moves from 36 mm before to 29 mm tension during the anaesthesia, a displacement in the same direction as in the previous experiment. — The coefficients of excitability are 0.84 and 0.45 for a waking state and for anaesthesia respectively.

It is remarkable, further, that the excitability in a waking state in this case is very low, the coefficient is at the same level as those seen earlier during sleep. This patient is the only one being psychotic (catatoniform schizophrenia), and there is no doubt that she has a pathologically low waking excitability (compare findings by melancholia [SCHOU, TROLLE and ØSTERGAARD 1942]).

If the results of the experiments are put together and arranged according to decreasing coefficients of excitability, three entirely separated groups appear. One is formed by the coefficients for 5 waking determinations with the values 1.64, 1.57, 1.41, 1.38 and 1.33. The next group consists of three sleeping coefficients, 1.13, 1.10 and 0.81, the two first from spontaneous sleep while the third is from the experiment with the deeper evipan sleep.

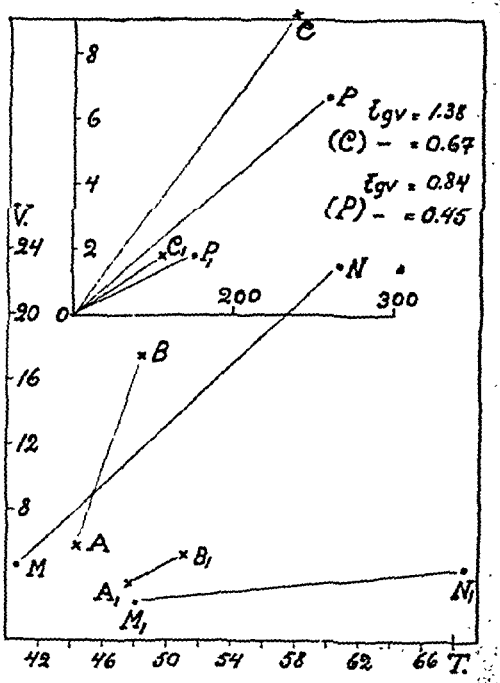


Fig. 5.

AB and OC curves of excitability for a waking state, A_1B_1 and OC_1 for anaesthesia from the experiment fig. 4. — MN and OP and also M_1N_1 and OP_1 corresponding curves from experiments on another subject (Q. B., 56 years).

In the middle of this "sleeping group" is a coefficient (0.84) from the waking determination in the patient last mentioned, in whom the excitability of the centre is considered pathologically low. — At the bottom of the scale a small "anaesthetic group" is formed by the two anaesthesia coefficients 0.67 and 0.45.

Discussion.

During evipan anaesthesia, even during light anaesthesia, the respiration is affected in a pronounced degree — the excitability of the respiratory centre towards CO_2 is considerably reduced.

The effect is far more pronounced than found in the experiments during sleep. The statement of WEESE and others, that the respiration is not or only very slightly affected during an ideal anaesthesia must therefore be considered disproved. The effect finds its expression in a reduced ventilation and in a rise in alveolar carbon dioxide tension. It is to be noted, that the ventilation during anaesthesia is relatively more reduced during CO_2 breathing than during atmospheric breathing, and for this reason the curves of excitability (ad modum LINDHARD 1933) during anaesthesia change direction besides being displaced depressively. This is in contrast to facts during sleep, where a parallel displacement is occasionally seen. Conditions during anaesthesia correspond to those mentioned by BEECHER and MOYER in experiments on dogs, that large CO_2 doses in the air breathed increase the reduction in the excitability of the respiratory centre caused by evipan.

In the case of anaesthesia the fall in ventilation is brought about by decreases both in frequency and depth of respiration. In the sleep experiments on the other hand the fall is first and foremost contingent on decreasing frequency. The "classic" statement, that the depth is increased during sleep, is not supported by the present results.

Owing to the considerably reduced respiration during evipan anaesthesia it is important that the evipan is administered very carefully. It appears from the curves, that the fall in ventilation is increased gradually by increasing depth of anaesthesia, which corresponds to findings in the experiments during sleep. The reduction in the excitability of the respiratory centre is consequently increased in proportion to the depth of anaesthesia, i. e. to the concentration of evipan in the blood. — At a certain concentration the centre as the first of the vital ones will be put out of function, and a paralysis of the respiration sets in. This most often happens by a sudden overdosing.

Summary.

Determinations are described of the excitability of the respiratory centre during a waking state, during spontaneous sleep and evipan sleep and during evipan anaesthesia by studying the reactions to the breathing of CO_2 mixtures (LINDHARD 1933).

2 experiments with spontaneous sleep show in both cases reduced excitability in the respiratory centre during sleep. 3 experiments

with sleep after giving of evipan in hypnotic doses show a larger reduction in the excitability which is solely due to deeper sleep, as it can be proved, that the evipan in hypnotic doses does not affect the centre depressively. From this the conclusion is drawn, that the reduction in excitability during sleep increases in proportion to the depth of the sleep.

Finally 8 experiments with evipan anaesthesia show that the excitability of the respiratory centre, even during light anaesthesia, is affected far more in a depressive direction than during sleep. The results of the experiments further show that this depression is increasing with the depth of the anaesthesia — i. e. with the concentration of evipan in the blood. At a certain concentration — as a rule this occurs by a sudden overdosing — the centre as the first of the vital ones will be put out of function, and the respiration paralysed.

During sleep ventilation was decreased 20—38 %, during anaesthesia falls of 48—80 % appeared. While during sleep the percentage fall occasionally was about the same with atmospheric and CO_2 breathing, a proportionally larger fall was caused by CO_2 during anaesthesia.

The tension of carbon dioxide in alveolar air rose during sleep 3—6 mm, during anaesthesia up to 8—9 mm. The fall in ventilation during sleep was first and foremost due to a reduction in frequency, during anaesthesia on the other hand both to reduction in frequency and in depth of respiration.

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Elasticity, Viscosity and Plasticity in the Cross Striated Muscle Fibre.

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In a former investigation (BUCHTHAL 1942) we have dealt with elastic properties of the isolated cross striated muscle fibre. Its *static elasticity* was determined from length-tension diagrams, and its *dynamic elasticity* and damping (viscosity) were analysed in vibration experiments using an oscillation period of 5 cycles per second. *Viscous and plastic properties* were investigated by means of semi-dynamic length-tension diagrams.

A definite and simple correlation was found *in the resting fibre* between tension (t) on the one hand and elastic properties (st) expressed by stiffness on the other $\left(\frac{\Delta\,\mathrm{st}}{\Delta\,\mathrm{t}} = \mathrm{k}\right)$. *During contraction* conditions are more complicated. Isometric contraction may reveal both lower and higher stiffness when compared with the same tension at rest. When however, an isometrically contracted fibre is released during stimulation, contraction stiffness always exceeds stiffness of the resting fibre (referred to the same tension). Length-tension diagrams of the isometrically contracted fibre show that its elasticity $\left(\text{measured by stiffness, } \frac{\Delta\ \text{tension}}{\Delta\ \text{length}}\right)$ decreases during contraction when a certain critical tension is reached. From the gradient of release-contraction diagrams and the course of semidynamic length-tension diagrams it follows that this phenomenon is caused by a plastic lengthening of equilibrium length during contraction (*yielding*). Similarly dynamic stiffness is found to decrease suddenly when a certain tension

is exceeded, probably the same value of tension responsible for the yielding which appears in length-tension diagrams.

Plasticity during contraction becomes apparent statically by a change in gradient of length-tension diagrams and dynamically by decreased stiffness.

The aim of the present investigation is to analyse conditions causing yielding and determine their quantitative influence on contraction tension and stiffness. For this purpose a method is developed for continuous and simultaneous measurements of stiffness and tension at rest and during contraction.

Furthermore, these experiments have led to an investigation of relations between elastic and viscous properties in muscle by determining stiffness with periodic changes in length of different frequencies. Formerly a mechanical equivalent circuit was derived from the time course of elastic after-effects and the damping constant, which represented a further development of LEVIN and WYMAN's (1927) diagram for total muscle. Here, with improved technique, an attempt is made to re-investigate the quantitative basis of such an equivalent and to determine temperature dependence of factors entering the equivalent diagram.

Method.

1) *Preparation.* The experiments are performed on single fibres or small bundles of m. semitendinosus (Rana esculenta and Rana temporaria) which are prepared from tendon to tendon in an ice-cooled Ringer solution of p_H 7.3. Oxygen content and constant p_H are ensured by passing a stream of 99 per cent O_2 and 1 per cent CO_2 through the solution. To establish a suitable colloid-osmotic pressure the Ringer solution contains, besides salts and glucose, 1.35 per cent Polyviol Am. Curarised muscle fibres are used in a number of experiments (0.5 μg pure curarine (KING) per g frog).

2) *Stiffness determination.* Stiffness is determined by continuous measurement of tension changes in the fibre originated by periodic alterations in its length. The latter are produced by an electro-magnetic system (Fig. 1, and Fig. 2 a_1) connected to a stainless steel micro-tweezer (2, Fig. 1)which can be moved in the direction of its longitudinal axis. The electro-magnetic device consists of the movable system of an ink writer unit used in electro-encephalography (BUCHTHAL and KAISER 1943).

Excursions of the tweezer (2, Fig. 1) are measured by transformation to capacity changes. These are recorded over a high frequency circuit (Fig. 2 a_4) with amplifier and electrostatic oscillograph, the deflections of which are proportional to the movements of the tweezer arm. One

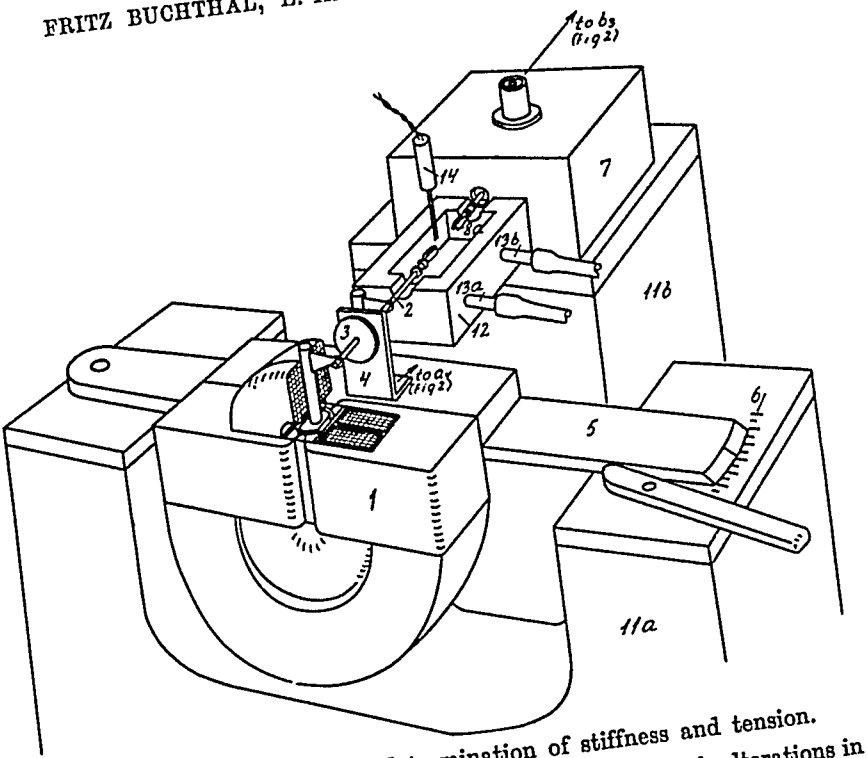

Fig. 1. Arrangement for determination of stiffness and tension.

1. electro-magnetic system for production of periodic length alterations in the fibre. 2. micro-tweezer for transference of length alterations. 3. and 4. condenser for determination of amplitude of length alterations. 5. movable arm carrying system 1—4 by which large changes in length can be performed. 6. scale for reading size of length alterations performed by movement of arm 5. 7. condenser-myograph (8, 9 and 10, detail fig. 3). 8 a. micro-tweezer for transference of tension to 7. 11 a. and b. cement blocks for the respective placing of length alteration and tension registering arrangements. 12. chamber with Ringer solution in which the fibre is placed between the tweezers. 13 a. and b. allow water of different temperature to pass through the walls of the chamber. 14. thermoelectric needle.

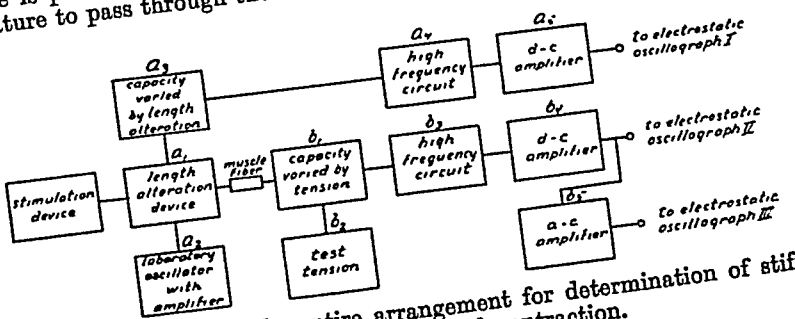

Fig. 2. Block diagram of the entire arrangement for determination of stiffness and tension during rest and contraction.

plate of the condenser (3) is fixed while the other is attached to the arm of tweezer (2) which holds one tendon end of the muscle fibre. The electro-magnetic system receives its energy from a laboratory oscillator with a maximal output of 4 watts. The amplitude of the periodic

changes in length is constant from 0 to about 300 cycles per sec but a maximal output of the oscillator would allow stiffness determinations with frequencies of up to 500 cycles per second. For low frequencies limits are set only by the oscillator which can be used for frequencies as low as 0.5 cycles per sec. When still lower frequencies are wanted the laboratory oscillator is replaced by a purely electro-mechanical arrangement. To obtain high measuring accuracy when determining amplitudes of these low frequency oscillations, movements of the mechanical alternating current generator are connected directly with the paper transport of the recording camera so that for any frequency the distance between oscillations is the same. Frequency is varied by changing recording velocity continuously and is measured by simultaneously registered time marks. The frequencies examined with this arrangement lie from 0.3 to 10 cycles per sec. When using frequencies above 50 cycles per sec the shape of curves should be as regular as possible. Superposed oscillations, which may occur accidentally are propagated faster than the main vibration and thereby give rise to phase displacements affecting the accuracy of stiffness determination.

When frequencies above 150—200 cycles per sec are applied the periodic tension variations which are superposed over an 8—10 mm long fibre are essentially deformed. The cause is the following: As stiffness increases proportionally with tension, a tension maximum in a vibration curve is propagated more rapidly than a tension minimum. Corresponding deformations occur when sinus-shaped oscillations are transmitted through tubes whereby oscillations become more jagged. The use of short fibres and small vibration amplitude will reduce these distortions occurring at high frequencies. Fibre lengths below 2—3 mm, however, introduce other difficulties, partly because a determination of absolute tension values becomes difficult (see page 21) partly because the deformation due to length alteration is different in the different parts of the fibre. The fibre zone nearest the tweezer will appear stiffer as variations in cross-section here are counteracted by its attachment. The shorter the fibre in proportion to the fastening zone the more important is the influence of the latter. Therefore, when investigating stiffness at high frequencies 4—5 mm long fibres are used and a saw-tooth vibration.

The superposed periodic tension variations should not exceed 20 per cent of extra-tension. Higher amplitudes increase the chances of yielding and lead to sudden alterations of tension and stiffness. When fibre bundles are worked on they must not contain tendon sheaths or tendon tissue shunting the contractile substance. The implication of shunting tissue leads to high and almost constant stiffness values as the stiffness variation of the contracting substance proper becomes relatively of minor importance.

Large variations in length i. e. those used for release or extension, are performed by moving the whole electro-mechanical system by lever (5) (Fig. 1). The size of these variations can be read on scale (6) or may be recorded using an electrical transmission.

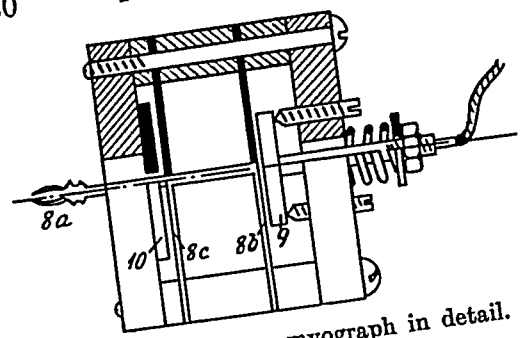

Fig. 3. Condenser-myograph in detail.

8 a. Micro-tweezer. 8 b. and c. phosphor-bronze springs to ensure parallel movement of 8a. 9. adjustable condenser electrode which with 8b forms a condenser. 10. condenser electrode with electric field for mechanical calibration.

3) *Measurement of tension.* Fibre tension is measured by means of the condenser myograph (8, Fig. 1) given in detail in Fig. 3. The other tendon end of the fibre is held by tweezer (8a) which is firmly attached to the one plate of a condenser. Capacity changes are used as measure of tension. They are transformed by a high frequency circuit (b_3 Fig. 2) and recorded by an oscillograph in connection with d. c. amplification when measuring static tension and with an a. c. amplifier and higher amplifica-

tion for tension variations. Opposite plate 8 b, a condenser electrode (9) is arranged at adjustable distance. Electrode and plate 8 a form a condenser the capacity of which depends on the axial load of the tweezer. Another condenser plate (10) is attached opposite plate 8 c to which electric tension can be introduced. By this means reproducible mechanical forces are obtained and applied to calibration of the myograph. Calibration tensions from 5—800 mg are produced by electric tensions of about 100—1200 V (mechanical tension varies with square of electrical tension). To ensure the undistorted registration of fast tension variations the movable part of the condensor consists of stiff plates of phosphor bronze (0.6 mm thick, 8 b and 8 c, Fig. 3). These plates have a distance of 15 mm, they guarantee a purely parallel movement of the tweezer and prevent registration of side pressure as tension. The condenser myograph has constant sensitivity for frequencies between 0 and 800 cycles per second, depending on the distance between the condenser plates and the amplification. Here sensitivity is not limited by the degree of amplification, but only by errors introduced through mechanical and acoustic disturbances. These are reduced as far as possible and direct transmission of vibrations to the tension recording apparatus is avoided by placing the latter and the electro-mechanical system on separate cement blocks, the only connection between them being the muscle fibre. There is a possibility that length variations are transmitted acoustically through the Ringer solution and received by the condenser myograph. Control experiments without fibre ensure, however, that this error is below the measuring accuracy. Each cement block is placed on a soft insulite plate which in turn lies on an elastically placed cement plate. The noise level of the arrangement corresponds to 1 mg.

Vibration amplitude (length alteration), tension and tension variation are recorded simultaneously by separate oscillographs. In some experiments a fourth oscillograph registers large length alterations or marking of stimulation.

It is necessary to control the measuring arrangement for possible non-linearities viz. oscillograph deflections do not vary proportionally with input tension. For correction it is immaterial if this non-linearity is due to the oscillograph itself or to distortions caused by other parts of the measuring circuit provided that test tensions controlling the entire circuit are introduced. Constant mechanical tensions of different size (a) superposed by constant alternating tension (b) allow control of sensitivity for different amplitudes. Alternating tension amplitudes (b) as function of tension (a) may serve as measure of relative sensitivity.

4) *Stimulation.* The stimuli applied consist of rectangular current pulses produced by a thyratron generator. Their duration is 1 ms and their frequency, when initiating tetanic contraction, 40—50 stimuli per second. The stimuli are applied to the fibre over the two stainless-steel tweezers 2 and 8 (Fig. 2).

5) *Variation of temperature.* The fibre is placed in a double-walled chamber of thin silver plates filled with Ringer solution. The temperature in the chamber can be changed by passing water of different temperature through the space between the silver plates and temperatures are continuously verified thermo-electrically.

6) *Measurement of absolute tension values and equilibrium length.* The influence of amplifier drift on absolute tension values is compensated for by frequently releasing the fibre from its instantaneous length to tension zero. The tension *difference* recorded corresponds to the fibre tension in the respective state. During this relaxation the fibre hangs relaxed in the Ringer solution in order to prevent tension development due to elastic after-effects. In this way absolute tension is measured in fibres of medium length with great accuracy. When fibre length however is below 2—3 mm the fastening zone in the tweezer will prevent it swerving sidewards and the otherwise distinct disappearance of tension around equilibrium length does not occur.

Equilibrium length is measured either by determining the length where tension just develops with an additional elongation or by determination of the fibre length where it is just straightened out. Both methods give identical results.

7) *Temperature dependence in the resting fibre* is measured by means of a horizontal spring balance where the tension displaces two parallel arranged spring plates. Their displacement is measured by a microscope with an eye-piece with movable cob-web. As tension is registered during several hours where unavoidable drift of the amplifier would imply corrections, this direct registration was preferred to the condenser myograph. Tension is measured with an accuracy of < 0.5 mg. All possible precautions are taken and control experiments performed to prevent erroneous results due to the influence of temperature changes on the measuring system. The fibre is held by two micro-tweezers consisting of Invar metal and in a series of experiments it rests on a quartz plate (Wöhlisch & Renk 1938, Wöhlisch 1943) to avoid the influence of updrift which varies with temperature. In every experiment a length-tension diagram is recorded. The fibre length, i. e. its equilibrium length, increases plastically due to the load applied over a long time and the

length measured at a given moment is no direct structural expression of the degree of stretch. Tension is a better expression and is used to evaluate the corresponding "structural" length from the length-tension diagram. As fibres with different cross-sections are examined, instead of comparing absolute tension values, the material is treated with regard to tension variation in relation to tension at high temperature. Tension and its changes are expressed logarithmically, whereby the relative tension changes can be read directly on the curve.

Results.

Stiffness and Plasticity during Contraction.

Experimental conditions used in the following experiments correspond largely to those formerly applied (BUCHTHAL 1942). The present method, however, allows a *continuous* determination of stiffness. To make full use of this advantage measuring frequency is increased from the formerly used 5 to 10 cycles per sec whereby a sufficient number of determinations with suitable time intervals can be obtained.

1. *Yielding in isometric contraction.* We have previously interpreted the decrease in contraction extra-tension with increasing elongation as being due to the limiting influence of tension on the propagation of contraction over the single molecule chains. This limiting influence is further indicated by the decrease of extra-stiffness during contraction with increasing initial tension, and contraction stiffness gradually approaches that of the resting fibre.

Stiffness determinations both at low (10 cycles per sec) and high frequencies reveal the dependence of contraction on initial stretch, and it is thought to be the result of a yielding process which *has* taken place. *In the following experiments an analysis of elastic properties during the yielding period itself is attempted.*

Tetanic contractions with superposed periodic tension variations (10 cycles per sec) are seen in Fig. 4. Contractions are released at different initial lengths and the amplitude of the periodic tension variations is a relative expression of fibre stiffness. At length 100 these tension variations are constant during contraction and indicate constant stiffness. Stiffness is reduced in the first phase of contraction initiated at length 108, a decrease which is still more distinct at lengths 116 and 125. At length 116

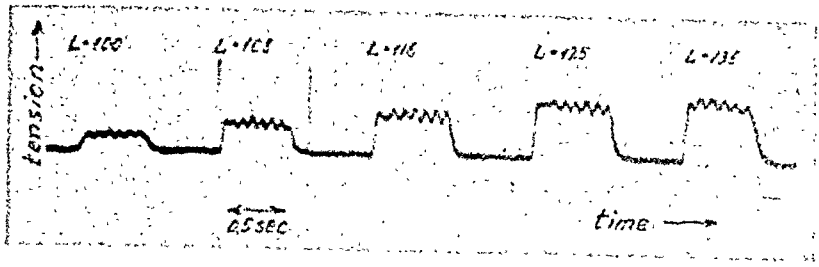

Fig. 4. Time course of tension in isometric tetanic contractions superposed with periodic changes in tension. Different initial lengths (equilibrium length = 100). Frequency of periodic changes 10 cycles per sec.

peak tension of the second oscillation is entirely lacking. At length 135 stiffness is constant but relatively low in proportion to tension. The low values in stiffness generally occur in the course of the first 0.5 sec of a tetanic contraction. Fig. 5 represents stiffness, tension and $\frac{\text{stiffness}}{\text{tension}}$ (F) for the example given in the preceding figure. Tension is relatively constant during contractions at the different elongations, while stiffness increases at length 108, 116 and 125 in the course of contraction. F shows corresponding variations. At length 100 F is highest (0.25), at length 108 it approaches this value slowly while at higher elongation it decreases and is 0.1 at length 135. *Thus, in spite of constant tension, contraction stiffness may vary considerably in a certain range of tension.*

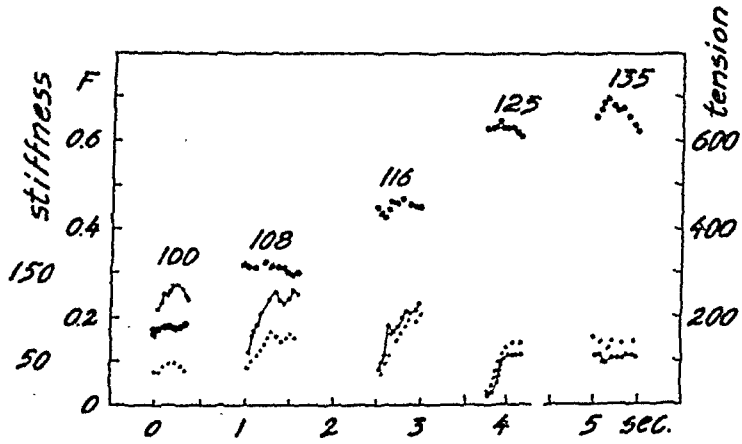

Fig. 5. Tension, stiffness and F as function of time in isometric tetanic contraction (calculated from original curves in fig. 4) at length 100 (equilibrium length), 108, 116, 125 and 135.

●●●● = tension, •••• = stiffness, ●—●—●—● = F.

2. *Yielding during length alterations of the tetanically contracted fibre.* The purpose of the following experiments is to find if there is any difference when tension, causing sudden changes in stiffness, is due to an intrinsic change in equilibrium length during contraction or to externally induced length alterations. Stiffness determinations are, therefore, performed during extension and release of the tetanically contracted fibre. Tension, stiffness and F as function of time during three extensions and releases of the continuously contracted fibre are seen in Fig. 6.

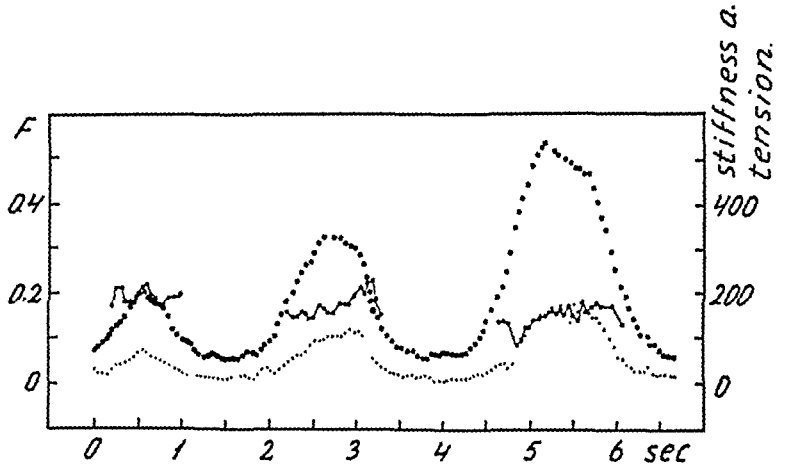

Fig. 6. Tension, stiffness and F as function of time. Three extensions during tetanic contraction.

●●●● = tension, •••• = stiffness, ●—●—●—● = F.

During the first stretch F is constant, in the next it is lower in the beginning and then increases, while during the last extension F is still lower, but increases again without, however, reaching the previous value. *The decrease in stiffness occurring at a certain tension level is thus independent of whether tension is due to internal changes in equilibrium length or to enforced variations in length.*

In Fig. 7 b—d the three extensions are represented as stiffness-tension diagrams. In diagram b (tension 60—200 units) extension and release are practically reversible. Stiffness during contraction is somewhat higher than at rest. In the next range of stretch (diagram c, tension 60—325 units), there is a clear difference between extension and release curves, stiffness becoming less during extension than during release as soon as tension exceeds 200 units. In diagram d tension reaches 530 units and above tension 200

there is a similar difference between extension and release. The gradient of both release and extension curves is lower in diagram d than in b and c. Due to yielding at high tensions, the amount of contracted substance is diminished and contraction values approximate those of the resting fibre. In resting fibres (diagram

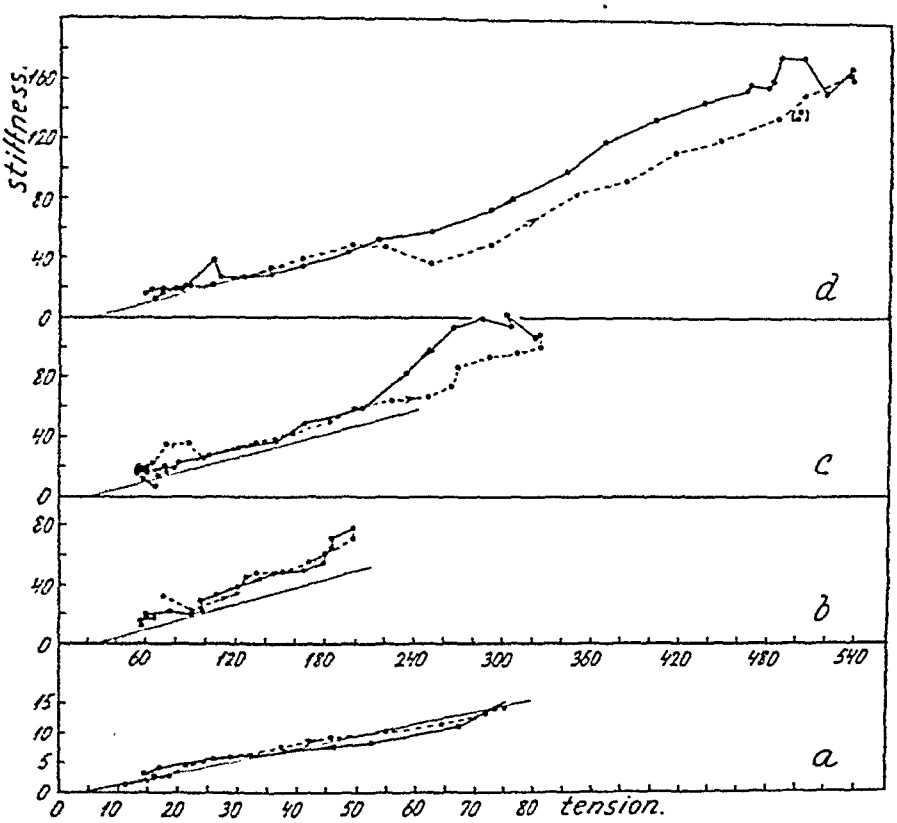

Fig. 7. Stiffness as function of tension.

a. rest. b, c and d. tetanic contraction with increasing degrees of extension during contraction. Due to the small tension and stiffness at rest the co-ordinate system in a is 4 times that in b—d.

●—●—●—● = extension, ● — ● — ● — ● = release, ——— = resting fibre.

a) the stiffness is reversible as function of tension during extension and release. *Stiffness-tension diagrams during contraction superposed with periodic tension variation of 10 cycles per sec thus show irreversibility when external tension exceeds a certain value, F being essentially less during extension than during release.*

3. *Comparison of stiffness determination at 10 and 100 cycles per sec.* Fibre tension is caused both by the reversible deformation and the force due to stiffness by which it counteracts a de-

formation. Reversible deformation denotes the difference between equilibrium length and the forced length of the fibre. When the latter is varied a change of reversible deformation and thereby of tension is originated.

The apparent decrease in stiffness accompanying yielding is probably due to corresponding changes in equilibrium length caused by the periodic alterations in length (irreversible deformations) which counteract the reversible deformation. When yielding

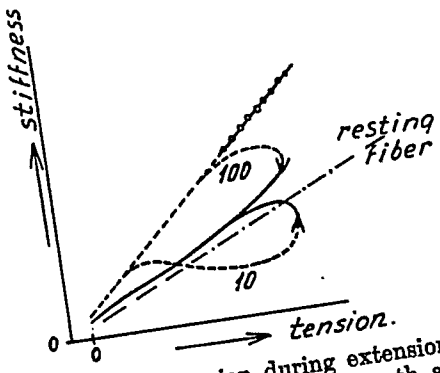

Fig. 8. Stiffness as function of tension during extension and release in tetanic contraction (schematic). Frequency of periodic length alterations 10 cycles per sec and 100 cycles per sec. The arrows on the curve show the time sequence of the measuring points.[1]

●—●—●—● = rest, — — — — = extension during tetanic contraction, ○—○—○—○ = course of stiffness as function of tension at 100 cycles per sec if all the substance remained in contraction. ——— = release during tetanic contraction,

increases, the effect of periodic length variations upon equilibrium length becomes more considerable and tension variations are decreased. *Due to yielding the stiffness measured thus appears lower than the true stiffness of the substance.* The induced changes in equilibrium length may in the initial phase of an isometric contraction follow almost completely the enforced periodic length alterations. This, however, is not the case when the frequency of length alterations is increased. Thus, when using a measuring frequency of 100 cycles per sec no measurable diminution of stiffness due to yielding can be observed. The rate of changes in equilibrium length would have to be increased 10 times to have the same influence as at 10 cycles per sec. The mean curve of all experiments using 10 cycles per sec shows that stiffness during

[1] Stiffness is always higher with 100 than with 10 cycles per sec. The difference which is due to viscosity is accounted for below. In the diagram we have disregarded this difference.

release exceeds that during extension by 10—15 per cent when tension amounts to 40—100 per cent of maximal contraction tension. As the decrease in stiffness during extension is due to yielding we may expect that stiffness determinations using 100 cycles per sec do not exhibit measurable differences between extension and release stiffnesses. Comparative experiments, however, reveal a variation opposite to that occurring at 10 cycles per sec, stiffness being 20 per cent higher during extension than during the release phase of contraction.

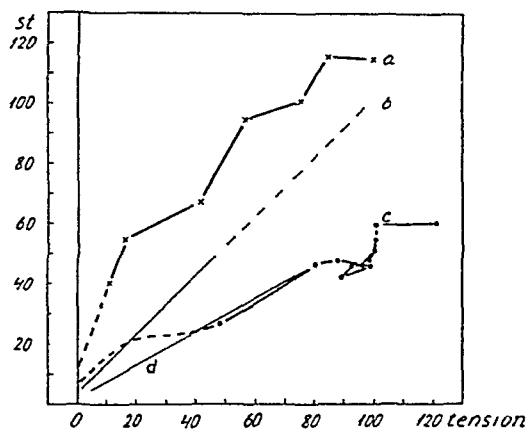

Fig. 9. Stiffness as function of tension during isometric tetanic contraction and at rest.

a. isometric contraction 100 cycles per sec. b. rest 100 cycles per sec. c. isometric tetanic contraction 10 cycles per sec. Contractions at low initial tension and low frequency were not registered in this experiment and the curve is extrapolated on the basis of other experiments using 10 and 5 cycles per sec.

Ordinate: stiffness in arbitrary units, Abscissa: tension in arbitrary units.

Yielding during extension occurs both when 10 and 100 cycles are applied, viz. due to the high stretch, part of the substance is pulled out of contraction. The release curve, common for both frequencies, is therefore in both cases nearer to the resting curve than the initial part of the extension curve (cf. schematic diagram Fig. 8). In the last two-thirds of the extension curve stiffness measured at 100 cycles per sec increases continuously with increasing tension as here yielding, though present, has no immediate influence. Using 10 cycles per sec a low stiffness is found in the same range of tension. During *release* F at both 100 and 10 cycles per sec is nearer the resting curve because yielding *has* occurred. Furthermore for 10 cycles per sec a decrease during *extension* is observed because yielding *is* occurring.

Stiffness-tension diagrams registered for the same fibre with both frequencies at rest and during contraction are seen in Fig. 9. With frequencies of 100 cycles per second contraction stiffness always exceeds that of the resting fibre while this is only the case at 10 cycles per sec as long as yielding has not entered.

4. *Measuring frequency and stiffness.* In the preceding section we have mainly dealt with the influence of yielding on stiffness measured with different frequencies. There is, however, another factor determining frequency dependence. Previous investigations have demonstrated elastic after-effects in muscle (v. KRIES 1880, BLIX 1892, GASSER and HILL 1924, H. H. WEBER 1941) and in single fibre (BUCHTHAL 1942). A sudden change in length causes higher increase in tension than would correspond to static stiffness. After this sudden increase tension decreases and approaches asymptotically the static tension belonging to the new fibre length (*consolidation*). The more rapid the length alteration the more the tension alterations exceed static values. *Dynamic stiffness, therefore, must be higher than static and will increase with increasing frequency.* Furthermore dynamic stiffness varies with consolidation velocity. With a high rate of consolidation, values of static and dynamic stiffness approach each other. *Increase in dynamic stiffness with increasing frequency thus is an expression of mutual relation between angular velocity of length alterations and rate of consolidation.*

Determination of static stiffness is difficult as the course of length-tension diagrams (the basis for static stiffness) depends to a large extent on the time used for their determination. Length-tension diagrams registered in the course of few minutes (semi-dynamic) exhibit about three times higher values of tension and stiffness than does a diagram registered in the course of one hour, where consolidation still goes on.

In the following sections experiments on dynamic stiffness as function of frequency at rest and during contraction are quoted.

Resting fibre. Stiffness referred to tension (F) as function of measuring frequency between 1 and 150 cycles per sec is represented in Fig. 10 c. The variation of F corresponds to the presence of a viscous shunt. In curve b_2 stiffness varies as the vectorial sum of an elastic and viscous stiffness, the latter dependent on measuring frequency. Curve c is a mean of experiments on slightly and moderately elongated fibres (lengths 120—150).

Stiffness as function of frequency varies less at higher and more at lower elongations than in the mean curve.

Between frequencies 0.5—10 cycles per sec no measurable variation of stiffness is found. On the other hand the difference in stiffness found between static experiments and those using 5 cycles per sec indicates that static stiffness is at least 50 per cent lower than stiffness at 5 cycles per sec. A considerable stiffness

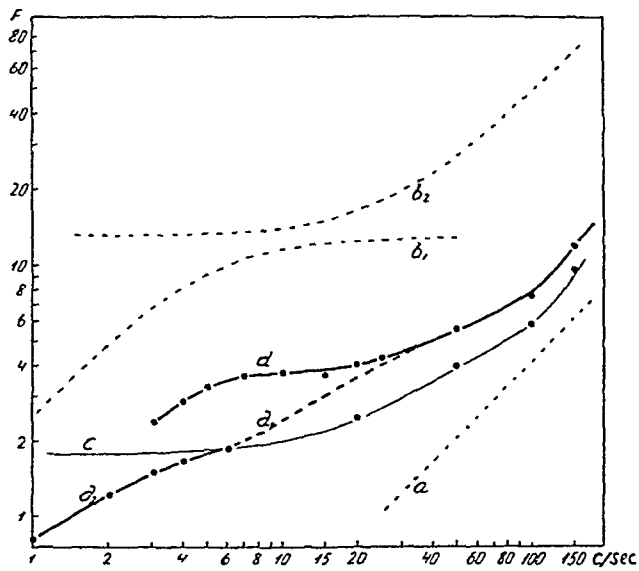

Fig. 10. Course of F $\left(\frac{\text{stiffness}}{\text{tension}}\right)$ as function of frequency of periodic length alterations introduced for the measurement of stiffness.

Frequency dependence
a. of the resistance caused by a viscosity against length alterations of constant amplitude. b_1 of a system consisting of elasticity and viscosity in series. b_2 of a system consisting of an elasticity shunted by a viscosity. c. of the resting fibre. d, d_1 and d_2 in isometric contraction (c. f. text). Ordinate: F. Abscissa: cycles per sec, logarithmic scale.

variation must therefore occur between 0 and 0.5 cycles per sec. A closer analysis of this frequency range will be attempted by experiments on elastic after-effects. The value of F at rest and its dependency on measuring frequency is not measurably affected by changes in temperature between 3 and 24° C.

Contracted fibre. Apart from the fact that stiffness during contraction exceeds that of the resting fibre between 20 and 150 cycles per sec the course of F during contraction corresponds to that at rest (Fig. 10, curve d). Stiffness during contraction varies however with temperature (cf. the following paper). When frequencies below 10 cycles are applied stiffness decreases essentially,

due to the fact that yielding becomes noticeable. We have analyzed this frequency range separately using an electro-mechanical alternating tension generator for low frequencies (see pg. 18). Curve d_2 shows F found in these experiments. Apart from a parallel displacement, the tendency of this curve is the same as in d. Stiffness during contraction reaches resting values at 6 cycles per sec. By extrapolating curve d the same would be the case at 2 cycles per sec. The difference is probably due to a more marked yielding in the last mentioned experiments and is in accordance with previous experiences (BUCHTHAL 1942) when frequencies of 5 cycles per sec were used, and where F during contraction could lie con-

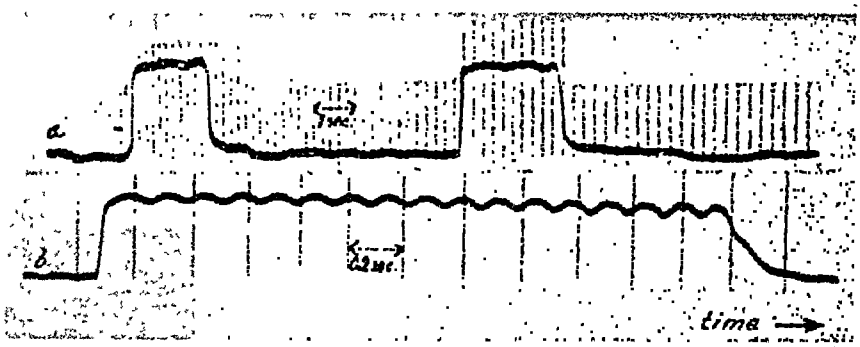

Fig. 11. Tetanic contractions with superposed periodic tension variations for determination of stiffness.

a. 0.5—1 cycle per sec. b. 7 cycles per sec. Distance between time markings 0.2 sec.

siderably above or below resting values depending on individual differences in the yielding effect of tension. Curve d_1 represents an extrapolation of d_2 to higher frequencies where the influence of yielding gradually decreases. A direct comparison of fibre stiffness at frequencies between 0.5 and 10 cycles, where contraction stiffness varies most with changes in frequency, is seen in Fig. 11. While frequency increases continuously, tetanic contractions (duration 2 sec) are released at suitable intervals (6 sec). During contraction at frequencies between 0.5 and 1 cycle per second amplitudes of periodic tension variations are small (low stiffness, Fig. 11 a) while the same length variation causes considerable periodic tension variations when frequency has increased to 7 cycles per sec (high stiffness, Fig. 11 b). Contraction tension is approximately identical at the different frequencies

Concerning the dependence of stiffness on measuring frequency we may summarize as follows: During isometric contraction stiffness decreases with falling frequency and attains, at low frequencies, values below those of the resting fibre. This behaviour is due to yielding, which has greater influence the lower the measuring frequency. Stiffness in release contractions, however, always exceeds resting values due to the occurrence of an elastic locking characterised by reversible length-tension diagrams and no yielding during vibrations (BUCHTHAL 1942).

The dependence of low frequency stiffness on temperature during isometric *tetanic* contraction[1] cannot be determined, as tension developed at low temperature is insufficient to produce yielding. Therefore comparisons between the effects of high and low temperatures become difficult. An investigation of contraction stiffness at low temperature and low frequency reveals higher values of F than at higher temperatures, but this increase may only be due to the fact that tension is too low to produce yielding.

5. *Temperature dependence of tension in the resting fibre.* Frequency dependence of dynamic stiffness is determined by the interaction of elasticity and viscosity. Taking into consideration that viscosity decreases with increasing temperature, it is surprising that dynamic stiffness at rest does not reveal measurable alterations with variations in temperature. On the other hand RENK and WÖHLISCH (1940) and MEYER and PICKEN (1937) find in the resting fibre changes in tension or length respectively when temperature is varied. The shortening tendency increases with *moderate* elongations and the opposite tendency is found at high degrees of stretch (50 per cent). At *slight* elongations there is disagreement, RENK and WÖHLISCH finding the same tendency here as at medium elongations while MEYER and PICKEN describe a second inversion in temperature dependence, the muscle behaving like all isotropic bodies and becoming longer with increasing temperature. RENK and WÖHLISCH have already drawn attention to the fact that the different thermal reaction of tendinous tissue may introduce errors when total muscles are applied and therefore use muscle strips. In connection with an investigation of temperature dependence of stiffness and viscosity we have examined temperature dependence of tension in resting fibres or

[1] Tension difference between high and low temperature is greater in tetanic contraction than in twitches. In the latter tension does not decrease noticeably until temperature is below 4° C.

bundles containing few fibres (Fig. 12). As the results in some respects deviate from the above mentioned investigations they are shortly quoted here (method cf. p. 22).

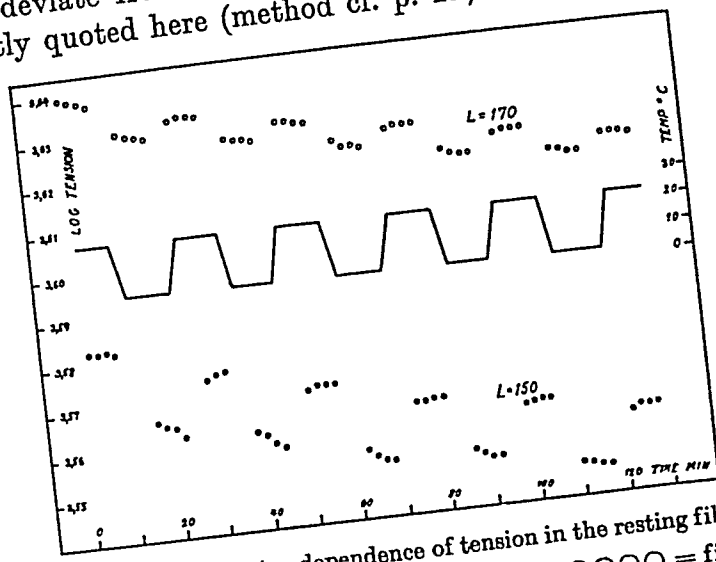

Fig. 12. Temperatur dependence of tension in the resting fibre. ——— = variation of temperature (right ordinate), ○○○○ = fibre tension, length 170, ●●●● = fibre tension, length 150.

Ordinate (left): log tension. Abscissa: time in min.

Fig. 13 shows the difference of log tension due to a difference in temperature of 25° C as function of stretch. The standard deviation is 1.2 per cent and the curve is determined by 24 points. Standard error in the different ranges of the curve amounts to approx. 0.5 per cent. In accordance with RENK and WÖHLISCH (1940) a shortening tendency is also observed at slight elongations, *while an inversion of temperature dependence does not occur at high*

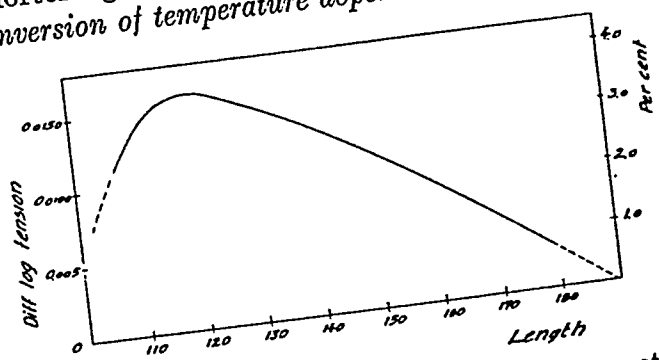

Fig. 13. Variation of difference in tension originated by a temperature difference of 25° C as function of length.

Ordinate (left): Difference in log tension; (right): tension variations in per cent. Abscissa: fibre length (equilibrium length = 100).

elongation. This difference in experimental results could be due to fascia sheaths or tendinous tissue present in the muscle strips used by RENK and WÖHLISCH. At high elongations the tendinous tissue would dominate the course of tension and its temperature dependence due to the low extensibility. The compensating effect of tendinous tissue could likewise explain why RENK and WÖHLISCH find, at maximum, only half as great a temperature dependence as we do, when comparisons are made on the basis of length-tension diagrams.

In contradistinction to MEYER and PICKEN our results, with those of RENK and WÖHLISCH, indicate a temperature dependence which is less than proportional with absolute temperature.

Discussion.

Comparative measurements of elastic properties with different frequencies lead to the conclusion that high frequency (100 cycles per sec) is preferable for determinations of stiffness proper which may be considered a suitable gauge for the relative amount of contracted substance. At low frequencies stiffness values are masked due to yielding and the apparent decrease in stiffness may serve as an indicator of the yielding process. In release contractions, where yielding has come to an end and the fibre is elastically locked, true stiffness values for the respective frequencies are also obtained.

The occurrence of yielding may be of importance in *physiological muscular activity*. It enables a muscle fibre at a given excitation to develop approximately equal tension in a large range of stretch and it prevents ruptures when a muscle is excited at high initial load.

On the other hand yielding limits the shortening ability to about 30 per cent. Larger shortenings may be expected before fibre tension has reached its critical value. In this connection mention may be made of former experiments (BUCHTHAL 1942), where muscular work increased through short interruptions of stimulation and elastic locking occurred at shorter fibre lengths.

Roughly speaking stiffness in the contractile substance may be of two different types, 1. structural stiffness and 2. "chemical" stiffness. *Structural stiffness* was treated in detail in a former investigation where stiffness as function of tension was analysed for spiral structures of molecule chains. As far as structural stiff-

ness is concerned an increase in stretch will cause an increased orientation, at first implying the adjustment of the fibre as a whole, then coarse micellar orientation and finally an increased orientation in minute structure.

The minute structural orientation, however, is not necessarily a purely mechanical process. In accordance with H. H. WEBER's (1934) assumptions length alterations in minute structure elements instead of being purely elastic may be accompanied by different adjustments of the molecule links dependent on tension (*"chemical stiffness"*). These structural modifications may by termed changes in linkage.

A mechanical equivalent circuit apart from pure elasticity contains elasticity shunted by viscosity. The frequency dependence of fibre stiffness just as the course of elastic after-effects indicates that viscosity is not uniformly distributed over the fibre. A mechanical equivalent however will become rather complicated if it is to contain factors accounting for both dynamic and static stiffness and their dependence on temperature. *Therefore, the assumption of changes in linkage appears more probable and viscosity is explained as being due to the velocity of transition from one linkage modification to another.*

On the basis of this assumption stiffness and viscosity at rest and during contraction and their temperature dependence may be interpreted as follows:

1. *Resting fibre.* The dependence of stiffness on frequency between 0 and 0.5 cycles per sec is supposed to be due to linkage modifications which occur slowly and effect changes in equilibrium length. Between 0.5 and 10 cycles per sec stiffness is constant, and might be due to purely elastic factors or to linkage modifications which rapidly adjust themselves in proportion to the vibration period. For measuring frequencies between 10 and 100 cycles per sec stiffness increases with frequency. Here it behaves as though caused either by purely elastic stiffness shunted with viscosity or by linkage modifications, which due to the increasing frequency have decreasing time to be established. The low temperature dependence at rest is easier to explain by supposing linkage modification than by the occurrence of intrinsic friction ("fluid friction"). A direct determination of stiffness at high and low temperature revealed no measurable dependence on temperature. Isometric length-tension diagrams have a different course at high and low temperatures. Around equilibrium

length and at high elongations values coincide for different temperatures while at moderate stretch tension is higher at high temperatures. The tension difference may amount to up to 4 per cent when temperature varies 25° C. Apart from pure elasticity, stiffness is thus dependent on the possibility of transition from a shorter linkage modification to a longer or vice versa. This possibility is determined by the probability of a certain modification in a certain range of stretch and by the velocity with which a transition can occur. At low tension we may reckon with the probable occurrence of several linkage modifications, (caused by tension) where the shorter forms will mostly be represented. At higher tension the probability of the occurrence of short modifications and of the transition from short to long modifications is diminished. Stiffness due to linkage modifications therefore will be greatest at high loading. As the temperature dependence of tension observed in the *resting* fibre can be quantitatively explained by thermal agitation, the experiments give no possibility of deciding whether the equilibrium between the different linkage modifications can be affected by temperature or not.

2. *Contracted fibre.* Irrigularities in the course of stiffness during isometric contraction are due to yielding, e. g. an elongation of the contractile elements irreversible during contraction. In terms of our model conception (BUCHTHAL 1942) this means that different lengths of modifications may occur. The molecule consists of contracted links and stretched resting substance. When extension forces exceed those due to contraction, part of the molecule chain yields and is transferred to rest or to a long modification during contraction. This transition takes a certain time and, therefore, the yielding does not appear immediately at high frequency but becomes distinct at frequencies below 10 cycles per second. In contrast to the resting fibre where the velocity of change in linkage must be independent of changes in temperature, stiffness during contraction at high frequencies depends considerably on temperature. Temperature dependence during contraction may be due to "intrinsic friction" between contracting elements[1] or to linkage modifications occurring with different and highly temperature-dependent velocity.

[1] Liquid displacements due to different elasticity in the A and I substance and occurring during contraction have formerly been treated quantitatively (BUCHTHAL 1942) and their influence is of minor importance.

Transition from one modification to another may be released by accidental thermal movements and can act on any part of the molecule chain implying low temperature dependence (proportionality with absolute temperatures). During contraction there is an additional factor, viz. *the propagated chain reaction*. The difference in temperature dependence of stiffness between rest and contraction could thus be caused by a change in the time of propagation over the chain highly dependent on temperature.

Summary.

1. A new method is devised for continuous measurement of stiffness at rest and during contraction.

2. Tension increase in tetanic contraction is limited by a plastic lengthening of equilibrium length in the contracted fibre (yielding), the occurrence of which is most marked in the first 0.5 sec of tetanic contraction. Yielding is characterised by a change in the gradient of static length-tension diagrams and dynamically by a sudden decrease in stiffness occurring at a critical tension value. This tension may be due either to contraction or to tension induced externally in the contracted fibre.

3. Comparative determinations of stiffness with low and high frequencies of periodic length alterations show that stiffness proper is masked by yielding when low frequencies are applied while a frequency of 100 cycles per sec is well suited to measurements of actual stiffness.

4. In physiological muscular activity yielding ensures constant contraction tension over a large range of stretch.

5. Apart from yielding the increasing stiffness with increasing frequency is due to viscosity, not uniformly distributed over the fibre.

6. In the resting fibre no measurable dependence of stiffness on temperature can be observed at different frequencies (3—25° C). During contraction stiffness decreases essentially with increasing temperature.

7. Evidence is given that this frequency dependence of stiffness is due to "linkage modifications" in contractile elements and that intrinsic friction is quantitatively of minor importance.

8. The difference in temperature dependence of stiffness at rest and during contraction is probably due to the chain reaction

occurring during contraction, the propagation of which is highly dependent on temperature.

9. At all degrees of stretch, tension in the resting muscle fibre increases with increasing temperature. The temperature dependence is highest at medium elongations and is always less than proportional with absolute temperature.

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Factors determining Tension Development in Skeletal Muscle.

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The purpose of the present investigation is a differentiation of the mechanical factors causing tension in the cross striated muscle fibre. A necessary basis for such differentiation is the existence of a definite relation between *deformation* and *tension* of the elastic body. This relation in a normal elastic body within the limits of proportionality is as follows:

$$\textbf{Tension} = \textbf{deformation} \times \textbf{stiffness}.$$

Tension is measured in dynes, deformation in cm, and stiffness in dyne cm^{-1}. Deformation is defined as the difference between the actual length of the body and the length it will assume in an unloaded condition. In a non-contractile body deformation arises only when it is affected by forces which alter its form. According to this definition, deformation under isometric conditions will arise also in a *contractile* body with contraction, as a change now appears in the unloaded length of the body.

The above relation is valid for a normal elastic body only, where the length-tension diagram is linear and stiffness as a function of tension constant. Conditions are more complicated with highly elastic material, where the possible change in length

is much larger. The length-tension diagram here is curved and stiffness $\left(\frac{\Delta \text{ tension}}{\Delta \text{ length}}\right)$ is consequently different for the different sections of the diagram. When, however, we know the mutual dependence of stiffness and tension, deformation of the highly elastic body can also be determined. In a previous investigation (BUCHTHAL 1942) we found *a linear relation* between stiffness and tension in the *resting fibre* after the coarse mechanical adjustment was complete. With the methods employed, the occurrence of *yielding* during tetanic contraction masked the actual stiffness of the contractile substance and inhibited a determination of its true elastic properties. By introducing a higher frequency of the periodic length alterations used to determine stiffness, the influence of yielding is considerably reduced in this investigation. The method, furthermore, allows determination of stiffness within short periods, so that a continual measurement of stiffness and tension in single twitches and during development of the tetanic contraction can be achieved.

The aim of the present investigation, therefore, is on the one hand, a determination of stiffness and tension and their mutual dependence to make possible an estimation of the elastic deformation, and on the other hand, an investigation of the time course of these magnitudes under different conditions by which their influence on the resulting mechanical tension can be analysed.

Method.

Measurement of mechanical tension and dynamic stiffness.

The tension which arises when the isometrically placed fibre contracts is registered by means of a condenser-myograph previously described (BUCHTHAL 1942, BUCHTHAL et al. 1944). Here, as in the previous investigation, the tension changes caused by periodic changes in length are used for a continual measurement of stiffness $\left(\frac{\Delta \text{ tension}}{\Delta \text{ length}}\right)$. Tension and stiffness, measured simultaneously, are examined at rest, during single twitches, as the tetanic contraction develops, and during the tetanic contraction itself. The periodic variations in length have a frequency of 100 cycles per sec and are produced by a laboratory oscillator with an amplifier connected to the electro-magnetic device for length variations described in the preceding paper.

The mechanical tension waves arising in the fibre with contraction or length variation transmit themselves at a mean rate of 10

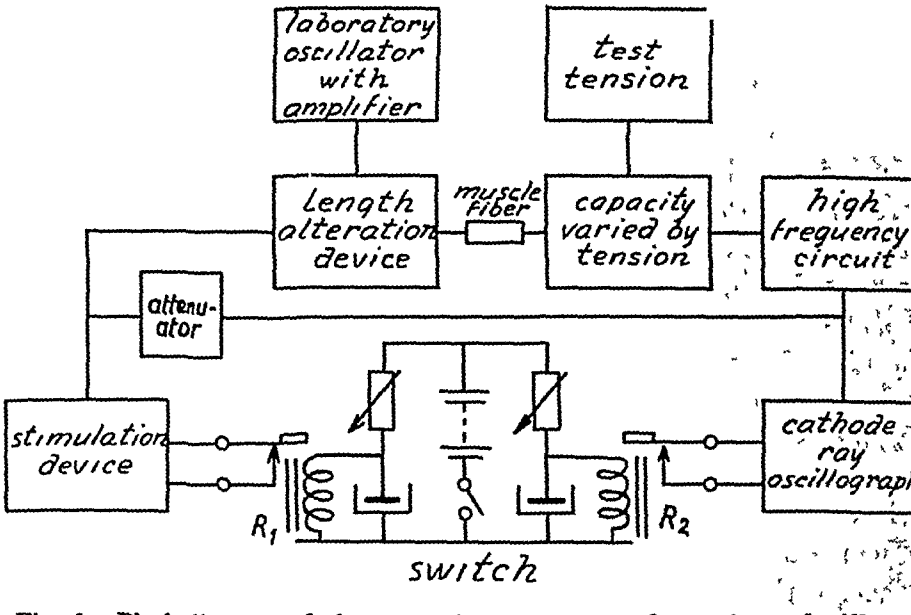

Fig. 1. Block-diagram of the measuring arrangement for tension and stiffness determination in the cross striated muscle fibre. Release of single stroke time base of cathode ray oscillograph and of fibre stimulation is performed by means of relays (R_1 and R_2).

m/sec over the fibre.[1] For a fibre 10 mm long, this means a maximal delay in the tension waves of 1 ms. In the simultaneous measurement of tension and stiffness this delay is of no practical importance, but must be taken into account when determining the latent period of the mechanical reaction. When investigating tension and stiffness during single contraction we are especially interested in their rapid variations, which are transformed from changes in capacity to changes in electric tension and are recorded by a cathode ray oscillograph and a condenser-coupled amplifier with a single stroke time base (camera: Contax, lens: Astro-Ardenne special: F: 1). With a rectangular voltage input the oscillograph deflection reaches 90 per cent of its maximum in 0.1 ms (appr.) and declines to $^1/_2$ in 800 ms. When using a cathode ray oscillograph, measurements of stiffness give no absolute values, but only the percentage variation of the relative stiffness during different conditions e. g. stretch, contraction, etc. Stimulation and start of single stroke is worked from the same mercury switch (Fig. 1, "switch"). When comparing rest and contraction it is necessary to record simultaneously a suitable section of tension and stiffness at rest with the course of tension during contraction and to mark the moment of stimulation on the film. By means of two relays (R_1 and R_2, Fig. 1) stimulation and start of the single stroke time-base can be varied ± 25 ms, so that the start of contraction can be focussed on the desired place

[1] Transmission of these mechanical tension waves must not be confused with the propagation of the contraction wave proper, which takes place at a rate of 0.3 m/sec, 24° C (Buchthal and Knappeis 1943).

of the fluorescent screen. Tension to R_1 and R_2 is led over a variable resistance and an electrolyte condenser.

The length of fibre with just sufficient increase to make tension measurable gives its *equilibrium length*. The *absolute tension* of the fibre — corresponding to the different fibre lengths — is measured by complete relaxation. The resulting difference in tension is a gauge of the fibre tension in the respective conditions.

For a further analysis (see page 47) of the mutual relation between stiffness, tension and elastic deformation, it is necessary to know the negative tension corresponding to stiffness zero. This tension, termed *stiffness-tension* (b), is deduced from stiffness-tension diagrams by extrapolation. When determining stiffness as a function of tension, length alterations of constant amplitude and a frequency of 100 cycles per sec are superposed over the fibre and serve as a measure of stiffness while fibre tension is varied by slow changes in length. From corresponding values of stiffness and tension, stiffness-tension diagrams are constructed for different fibre conditions. As these are practically linear, stiffness-tension (b) can thus be determined by extrapolation to stiffness zero. To obtain the greatest possible accuracy, a series of these stiffness-tension diagrams are always recorded and their mean gradient used to determine (b). The error introduced by extrapolation is discussed with the other errors in measurement, (page 49). Just as with the electrostatic oscillograph (BUCHTHAL et al. 1944) *control of a possible non-linearity* (output not varying proportionally with input tension) is necessary. The sensitivity of the cathode ray oscillograph varies in the direction of the y-axis as well as a function of the size of deflection and of the position of the light spot on the x-axis. Different constant mechanical tensions superposed by alternating tensions of constant amplitude are recorded as control. To facilitate the necessary two-dimensional correction, the size of deflection caused by alternating tensions is measured as a function of the position of the light spot on the screen (caused by constant tension). Points with the same sensitivity (same deflection size) have the same correction and are connected. These curves are drawn on transparent celluloid on the same scale as the enlarged oscillograms, and by placing the celluloid plates on the latter, the correction for different points of the oscillogram can be read directly.

Due to the time constant of the amplifier, a further correction proportional to the size of the extra-tension and duration of the contraction, but applying only to the relaxation period of single contraction, is necessary. For a twitch with a duration of 80—100 ms the correction at the final phase of contraction is about 2 per cent of the extra-tension developed.

Measurement of the Oscillograms.

The oscillograms are enlarged photographically 4—5 times. On the enlargement the positive and negative peaks of the periodic vibrations are marked by puncturing, and the holes (enlarged 5 times) projected

on millimetre paper. By connecting the projected points — upper and lower curve of the periodic tension variations respectively — we get an envelope curve. The envelope amplitude is a measure of the relative stiffness. The middle between the upper and the lower curves gives the course of the extra-tension proper. At least four curves are projected on the same millimetre paper; they are recorded immediately after each other and synchronised with reference to the moment of stimulation. As the periodic tension variations identical in the four experiments are not synchronous, the resulting curve contains more points per time unit, and its time course, therefore, is determined with more exactitude. With a measuring frequency of 100 cycles per sec on the single envelope curve the distance between two points amounts to 10 ms. The average distance in the mean curve should become 2.5 ms. One can, however, only reckon with an effective point distance of approx. 5 ms due to the random distribution of the measuring points. The technique for measuring oscillograms described here is easier and proves just as accurate as measurement by a measuring microscope.

Preparation and stimulation.

The experiments are performed on intact isolated fibres or small bundles without tendon sheaths or fascia of m. semtendinosus (Rana temporaria and Rana esculenta). The preparation is placed between two stainless steel micro-tweezers (cf. Fig. 1 BUCHTHAL et al. 1944) in Ringer solution of pH 7.3 the temperature of which is controlled thermo-electrically.

Stimulation is produced by a thyratron generator and consists of rectangular pulses with a duration of 1—0.2 ms. The stimulus is led to the fibre over the two micro-tweezers and its relative size is registered by leading part of the stimulus tension over a high resistance to the cathode ray oscillograph. When investigating the initial course of single contraction it is important to apply the shortest possible stimulus to avoid distortion of the curve, which would compromise stiffness measurements during transition from rest to contraction. A number of experiments are performed on curarised fibres (0.5 μg Curarine per g. frog).

Results.

An oscillogram of the mechanical tension in single contraction superposed with periodic tension variations for the determination of stiffness is shown in Fig. 2. The rising tension during development of contraction is accompanied by an increase of the envelope amplitude which corresponds to the relative stiffness. Fig. 3 shows the envelope curves for a single contraction. Before stimulation the upper and lower curves run parallel, corresponding to constant tension and constant stiffness. After stimula-

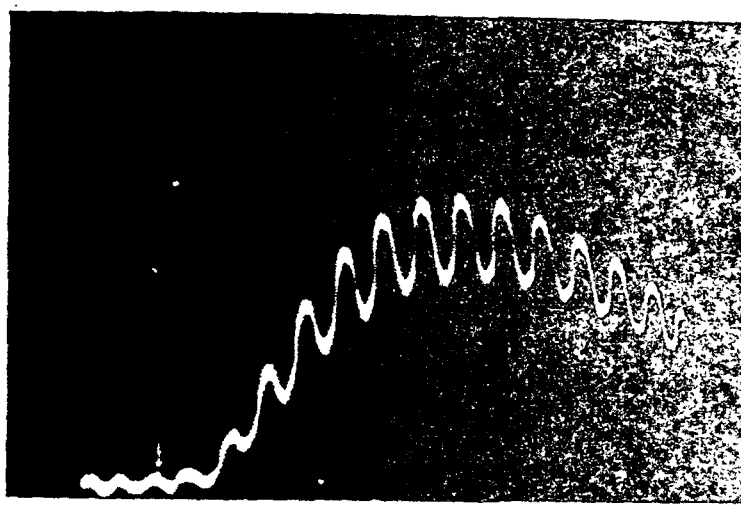

Fig. 2. Oscillogram of tension in a twitch superposed by periodic changes in length for determination of stiffness at a frequency of 100 cycles per sec, temperature = 15 °C. ↓ moment of stimulation.

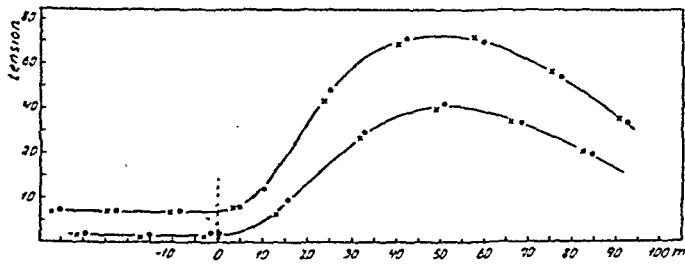

Fig. 3. Time course of envelope curves of the periodic tension variations (mean curve of experiment ● and experiment ×).

Ordinate: tension in arbitrary units.
Abscissa: time in ms.
Stimulation at 0.

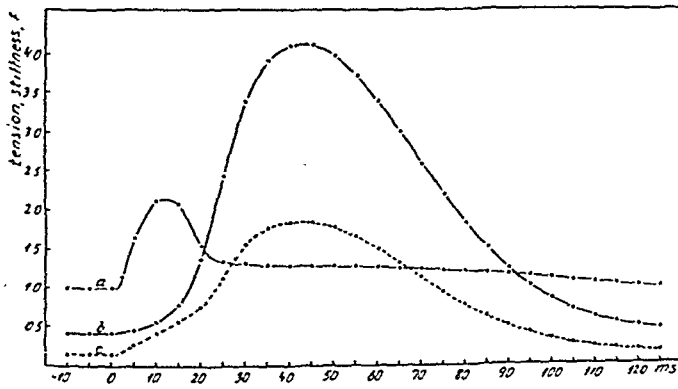

Fig. 4. Tension (b) stiffness (c) and F (a) as function of time.

Stimulation introduced at 0.
Ordinate: F, tension and stiffness in arbitrary units.
Abscissa: time in ms.

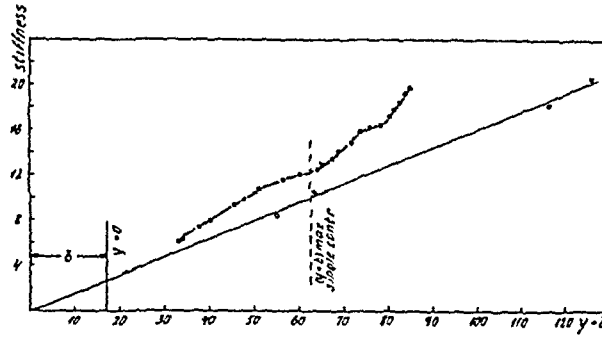

Fig. 5. Stiffness as function of tension at rest (-o-o-o-o) and during isometric tetanic contraction (—•—•—•)
Ordinate: stiffness in arbitrary units.
Abscissa: tension + stiffness-tension (b) in arbitrary units.

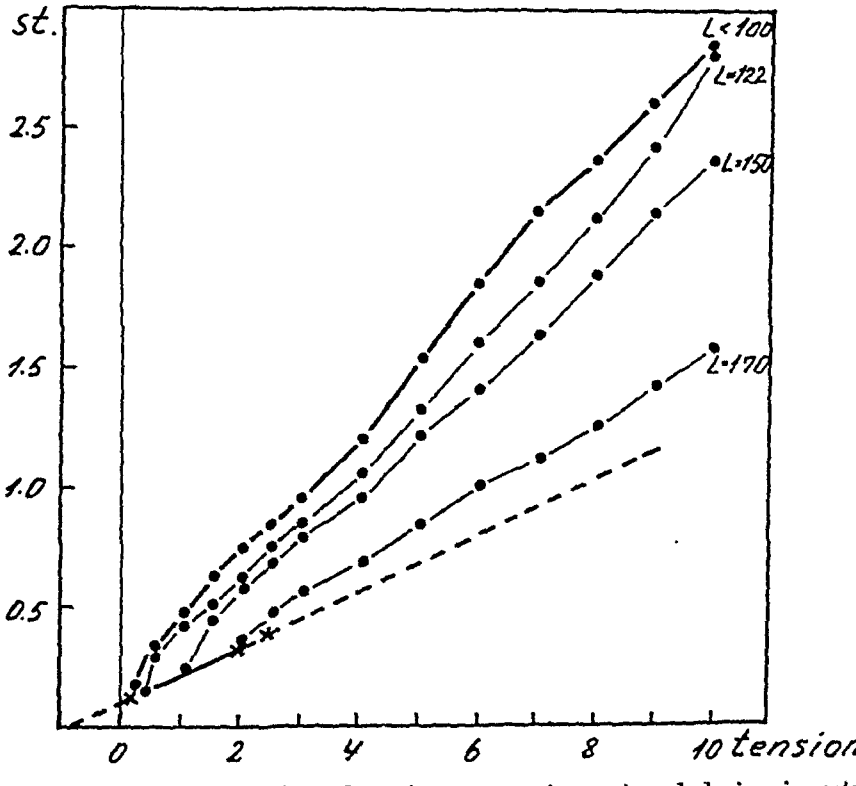

Fig. 6. Stiffness as function of tension at rest (- - - - -) and during isometric tetanic contraction initiated at different original lengths. In the top curve contraction is initiated at a length below 100 (equilibrium length = 100).
Ordinate: stiffness in arbitrary units.
Abscissa: tension in arbitrary units.

tion, when extra-tension develops, both curves rise. The mean value of the envelope curves corresponds to the course of extra-tension and the distance between them expresses variations of

stiffness during contraction. Tension and stiffness are corrected for non-linearity due to the measuring arrangement and the time constant of the amplifier, as previously described. The corrected time course of these magnitudes during contraction (Fig. 4) shows a striking similarity indicating a definite quantitative relation between stiffness and tension during contraction.

To examine this relation over the largest possible range of tension, stiffness is determined as a function of tension in the resting and tetanically contracted fibre, using an oscillation fre-

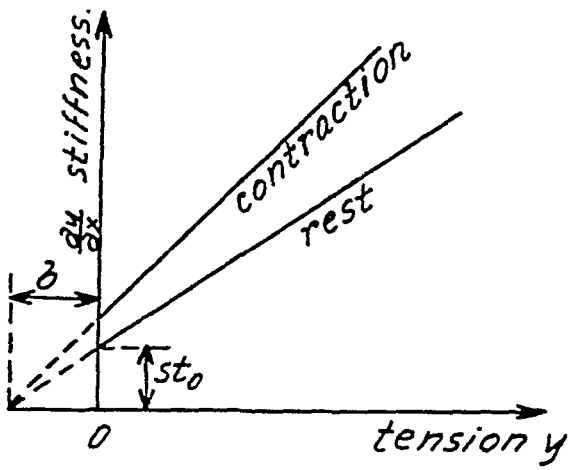

Fig. 7. Stiffness-tension diagram at rest and during contraction with extrapolation for determining of stiffness-tension (b) (schematically).
Ordinate: stiffness in arbitrary units.
Abscissa: tension in arbitrary units.

quency of 100 cycles per sec (Fig. 5). *Stiffness at rest varies proportionally with tension,* a result in accordance with former experiments where oscillation frequency was 5 cycles per sec. (BUCHTHAL 1942). Stiffness at rest is almost independent of the rate in tension changes, so that stiffness-tension diagrams have the same gradient for different rates of stretch. *Also during contraction an approximately linear interdependence between stiffness and tension is found* when using a measuring frequency of 100 cycles per sec, this frequency being so high that yielding is not noticeable (Figs. 5 and 6). *Stiffness referred to the same tension is higher during contraction than at rest* and is here somewhat dependent on the rate of changes in length or tension (due probably to the fact that the state of contraction can be altered by exterior influences). Fig. 6 shows stiffness-tension diagrams of a fibre in tetanic contraction initiated at

different original lengths. The difference between stiffness during contraction and rest decreases with increasing original length. Disregarding the transition from rest to contraction, stiffness in all fibre lengths increases proportionally with tension. During the transition from rest to contraction stiffness naturally increases more steeply as it occurs in a relatively short period, and as the contraction curve has a higher gradient and lies above the resting curve. Also, in the transition period from rest to contraction stiffness depends both on extra-tension and original length. With increasing elongation, the difference between rest and contraction decreases.

Stiffness measured in the present investigations is a *dynamic stiffness* and the conclusions drawn from these measurements can refer to dynamic properties only. A detailed differentiation between static and dynamic stiffness, as it is brought about when using different frequencies of periodic changes in length, is given in a previous paper (BUCHTHAL et al. 1944). Here mention is made only of the fact that measurements of elasticity with frequencies over 300 cycles per sec are unsuitable for an analysis of changes produced by the contraction process. With this frequency the difference between stiffness at rest and during contraction (referred to the same tension) is unmeasurable.

A frequency of 100 cycles per sec for periodical length alternations has proved well suited for determining dynamic stiffness. *With this frequency a simple quantitative dependence between stiffness and tension is found at rest as well as during contraction, which permits an estimation of the factors determining the development of tension in the cross striated muscle fibre.*

Stiffness-tension diagrams for the resting and contracting fibre are represented schematically in Fig. 7. While the length-tension diagram according to definition starts with tension zero, a certain stiffness corresponds to this tension implying the presence of a definite structure with, as well as without load. This initial stiffness is somewhat higher during contraction than at rest, so that rest and contraction curves intersect in the stiffness-tension diagrams when extrapolating to stiffness zero. The extrapolated negative tension (stiffness-tension (b) cf. page 41) is practically identical during rest and contraction.

From the linear dependence between stiffness and tension it is possible to form a simple common expression for the mechanical

properties at rest and during contraction. From the definition of stiffness it follows that

$$st = \frac{dy}{dx} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where st is stiffness, y tension and x change in length.

The linear dependence between stiffness and tension found experimentally can be expressed as

$$\frac{dst}{dy} = F = \text{constant} \quad \ldots\ldots\ldots\ldots \quad (2)$$

Experiments show further that stiffness-tension (b) is constant and (2) can be expressed by

$$st = (y + b) \cdot F \quad \ldots\ldots\ldots\ldots \quad (3)$$

Substituting (1) for (3) we get

$$\frac{dy}{dx} = (y + b) \cdot F \quad \ldots\ldots\ldots\ldots \quad (4)$$

By transposing, F becomes

$$F = \frac{\frac{dy}{dx}}{y + b} \quad \ldots\ldots\ldots\ldots \quad (5)$$

$$F = \frac{\frac{d\,(y + b)}{dx}}{y + b} \quad \ldots\ldots\ldots\ldots \quad (6)$$

By integration of (6) we get

$$\log (y + b) = Fx + c \quad \ldots\ldots\ldots\ldots \quad (7)$$

where c is an arbitrary constant.
As $y = 0$ corresponds to $x = 0$
we have $\log b = c$

$$\log (y + b) = F \cdot x + \log b \quad \ldots\ldots \quad (8)$$

$$\log (y + b) - \log b = F \cdot x \quad \ldots\ldots \quad (9)$$

$$\log \left(\frac{y + b}{b}\right) = F \cdot x \quad \ldots\ldots\ldots\ldots \quad (10)$$

$$x = \frac{\log \frac{y + b}{b}}{F} \quad \ldots\ldots\ldots\ldots \quad (11)$$

From (10) follows furthermore

$$\frac{y + b}{b} = e^{Fx} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (12)$$

and from this

$$y = (e^{Fx} - 1)\, b \quad \ldots\ldots\ldots\ldots\ldots\ldots (13)$$

where e is the base of the natural logarithms.

From equations (11) and (13) where b, y and F are known, *the deformation* x *can be found which expresses the dynamic shortening the fibre is capable of, when released to tension zero.* For tension development stiffness (F) as well as deformation (x) is necessary. When forming the equations, it is taken for granted that no deformation exists at equilibrium length at rest. The exponent to e, the product Fx, will thus be zero, $e^{Fx} = 1$ and consequently $y = 0$. Development of tension at this length presupposes a tendency to shorten caused by the contraction process. The deformation expressed by x denotes the difference between equilibrium length in the resting fibre and the new equilibrium length due to contraction. With greater lengths (deformation being present at rest and x having a finite value) tension can develop with a change in F only. The increase in stiffness in a stretched fibre can thus effect an increase in tension without a simultaneous change in the equilibrium length as the stiffer fibre tries with greater force to attain its equilibrium length.[1]

When we want to calculate dynamic equilibrium length i. e. the *absolute* dynamic contraction ability from relative values, the absolute values for at least one relative shortening must be known. In the present experiments we know the dynamic shortening of the resting fibre which amounts approximately to half the degree of stretch above the static equilibrium length (BUCHTHAL 1942). The shortening ability (Δ L) can therefore be expressed by:

$$\Delta L = \frac{\log \frac{y_{contr.} + b}{b}}{\log \frac{y_{rest} + b}{b}} \cdot \frac{F_o}{F} \cdot \frac{L - 100}{2} \quad \ldots\ldots\ldots (14)$$

where L is the length in per cent of the resting fibre (equilibrium length = 100).

[1] For the two fibre substances (anisotropic (A) and isotropic (I), this means that A yields a contribution to tension by a change in F as well as in x, and I only on account of the change in F (cf. BUCHTHAL 1942).

The work (W) performed by the fibre when released is obtained by integration of equation (13) with regard to x

$$W = \frac{e^{Fx} - 1}{F} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (15)$$

$$W = \frac{\frac{y}{b}}{F} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (16)$$

From equation (15) it follows that work is directly proportional to tension and inversely proportional to stiffness. Increasing softness will, with a given fibre tension, cause a greater shortening and consequently more work is performed. Work is naturally greater with higher tension. When the stiffness is infinite or the original tension zero, work will also be zero as the fibre cannot shorten.

Thus these equations permit a formulation of the factors determining the size of mechanical tension in the cross striated muscle fibre. It would be tempting to investigate the amount of tension contributed by each of these factors, were the task not impossible due to stiffness and deformation entering the equation exponentially. The contribution in tension of the one component can thus not be expressed independently of the other. A certain change in F will effect a higher change in tension when deformation is large than when it is slight. *The changes in stiffness referred to the same tension* (F) *and in equilibrium length as a function of time, however, show the influence of these factors on mechanical tension under different conditions.*

Estimation of Error.

Computing the dynamic shortening expressed in equation (14) uncertainties enter for the following magnitudes: b, y_r, y_c, F, F_o, and L.

1. Error in b. Extrapolation of the stiffness-tension diagrams to determine b is dependent on stiffness 0 (st_o) and on the gradient of the stiffness-tension diagrams at rest (F_o). Stiffness-tension b, which is a projection of the extrapolated section of the curve on the abscissa (Fig. 7) can be expressed by:

$$b = \frac{st_o}{F_o} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (17)$$

Error in b (Δ b) can therefore be expressed by means of the errors in st_o and F_o (Δ st_o and Δ F_o) which are generally 10 and 5 per cent respectively. The resulting error in b will be about 11 per cent and is

dominated by st_o as ΔF_o only increases the error about 1 per cent. We can, therefore, ignore the influence of ΔF_o on Δb.

2. *Error in determination of y (Δy) and of F and F_o (ΔF and ΔF_o)* generally amounts to 5 and 10 per cent respectively.

3. *Error in determination of length (L)* is maximally 2 per cent, which in the range of stretch from 120—160 gives an error (a) on $\frac{L-100}{2}$ of between 10 and 3 per cent.

The percentage influence of Δb and Δy upon $\log \frac{y+b}{b}$ for different values of $\frac{y}{b}$ between 0.1 and 100 is given in table 1.

Table 1.

$\frac{y}{b}$	0.1	0.5	1.0	2.0	3.0	4.0	9.0	100.0
$\Delta\left(\log\frac{y+b}{b}\right)\Delta b$	10	8	7	6	5	4.5	3.5	2
$\Delta\left(\log\frac{y+b}{b}\right)\Delta y$	5	4	3.5	3	2.5	2.3	1.8	1

The error resulting from b (β) on $\frac{\log\frac{y_c+b}{b}}{\log\frac{y_r+b}{b}}$ is the difference between the errors of the numerator and denominator and is between 2 and 4 per cent in the range measured. The error in the denominator (γ) caused by y amounts to from 2—4 per cent; the error in the numerator caused by y_c is dependent on the error in $\frac{F}{F_o}$ as $F = \frac{st_c}{y_c+b}$. This error must therefore be calculated in connection with the error in F determined by the error in stiffness ($\Delta st_c = 5$ per cent) and the error in tension ($\Delta y_c = 5$ per cent). As y during contraction is considerable, the error in b can be ignored. The influence of Δy_c on the numerator (δ) lies between 2.5 and 1 per cent and on F (Σ) between 8 and 2 per cent. The resulting percentage error in the *change* in length (ω) is therefore

$$\omega = \sqrt{\beta^2 + \gamma^2 + (\delta + \Sigma)^2 + \Delta F_o^2 + \Delta st_c^2 + a^2} \quad \ldots \quad (18)$$

By substituting the given maximal and minimal values we get:

$$\left.\begin{aligned}\omega_{max} &= \sqrt{5^2 + 4^2 + (2.5 + 8)^2 + 5^2 + 5^2 + 10^2} = \\ &= \sqrt{291} = 17 \text{ per cent} \ \ldots\ldots\ldots\ldots\end{aligned}\right\} (19)$$

$$\left.\begin{aligned}\omega_{min} &= \sqrt{3^2 + 2^2 + (1.0 + 2)^2 + 5^2 + 5^2 + 3^2} = \\ &= \sqrt{81} = 9 \text{ per cent} \ \ldots\ldots\ldots\ldots\end{aligned}\right\} (20)$$

The values found for ω denote the error in per cent of the *shortening*. With small lengths where shortening amounts to about 40 per cent, ω_{max} must be taken into account, and error in the length proper will be $0.4 \cdot 17 = 7$ per cent. With larger elongations where the size of error is determined by the ω_{min} and shortening amounts to about 20 per cent, the error in the length proper will be $0.2 \cdot 9 = 2$ per cent.

Tension, stiffness and dynamic shortening in single twitches are subsequently investigated as function of stretch, stimulation, temperature and degree of fatigue.

1. *The mechanical reactions of the fibre after maximal stimulation at different degrees of stretch.*

The time course of *extra-tension* during single contraction at different elongations is seen in Fig. 8 a. The extra-tension increases up to a length of 126, after which it decreases, a course which corresponds to that known from length-tension diagrams for single contractions. The simultaneously registered time course of *stiffness* referred *to the same tension* is given in Fig 8 b where F denotes the actual $\frac{\text{stiffness}}{\text{tension}}$ during contraction and F_o the corresponding value at rest. Shortly after the moment of stimulation $\frac{F}{F_o}$ increases steeply and in all cases has a maximum which occurs essentially earlier than the maximal tension. In the present example F_{max} occurs 20—25 ms after stimulation and the maximal extra-tension 50—60 ms after stimulation. With increasing stretch F_{max} is graded in the same way as the maximal value of tension. $\frac{F}{F_o}$ attains its original value within 100 ms at small degrees of stretch, while at medium and large elongations it can lie up to 20 per cent above the resting value in the relaxation phase of the contraction. *Increase in stiffness during contraction (referred to the same tension) in comparison to stiffness at rest can amount to 30—90 per cent depending on the degree of stretch.* Thus the contraction values for F usually lie above the resting values, but in the final phase of the contraction F is sometimes lower than F_o.

The course of the *dynamic shortening* calculated from tension and $\frac{F}{F_o}$ is shown in Fig. 8 c. Δ length denotes the difference

between the dynamic shortening ability at rest and during contraction, i. e. Δ length $= = \Delta L - \frac{100}{2}$ ..... (21), where ΔL is given by equation (14) pg. 48. At length 108 (equilibrium length at rest = 100) the dynamic shortening amounts to 3 units at rest. When contraction begins the dynamic equilibrium length decreases, (*i. e.* the fibre shows a tendency to shorten) and attains its minimum about 55 ms after stimulation. With this low degree of stretch the contraction maximum and the maximum value of extra-tension occur simultaneously. At L = 126 dynamic shortening at rest is 10 units, the curve course during contraction corresponds to that of L = 108. At mean elongations (e. g. L = 143) the dynamic shortening at rest reaches 23 units *and falls immediately after the stimulation* (15 ms after the moment of stimulation) to 18 units, after which

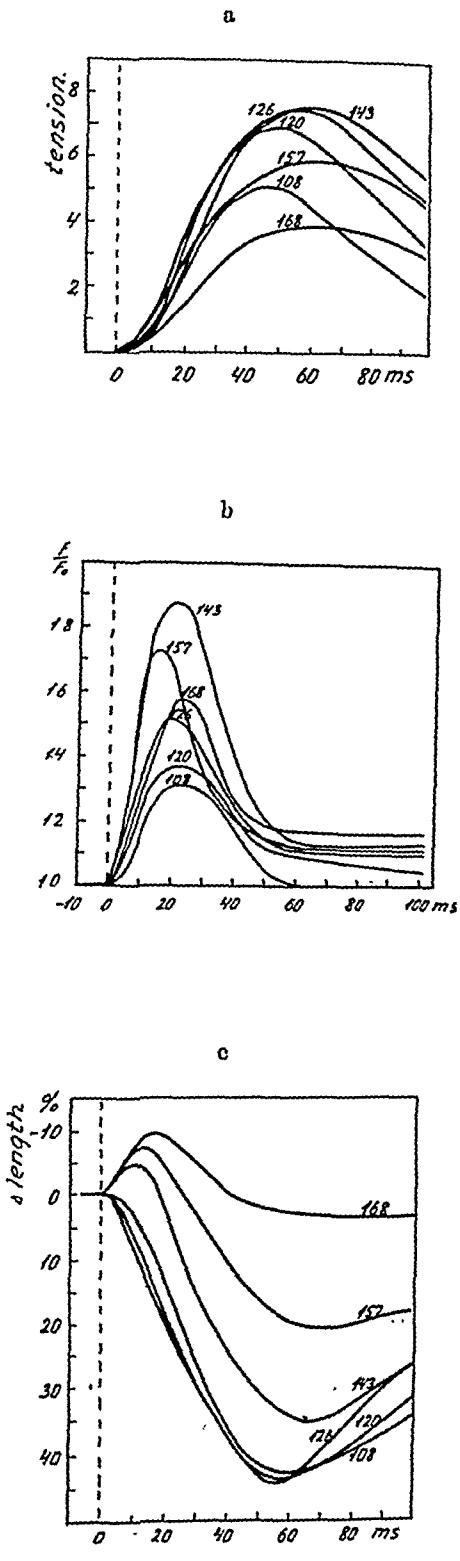

Fig. 8. Extra-tension (a), $\frac{F}{F_0}$ (b) and deformation (c) in single contractions at different degrees of stretch as function of time.

Ordinate: curve a: tension in arbitrary units.

curve b: $\frac{F}{F_0}$

curve c: Δ length (cf. equation (21)).

Abscissa (common for a, b, and c): time in ms.

Figures on the curves denote fibre lengths (equilibrium length at rest=100).

the normal contraction begins. Here the maximum of shortening occurs later than that of tension and is reached 65 ms after stimulation. At length 157 the rise in dynamic equilibrium length immediately after stimulation is 50 per cent higher than at length 143 and the dynamic shortening following is still less than before. The shortening maximum occurs about 70 ms after the maximal tension. At length 168 an increase in dynamic equilibrium length twice as large as that at length 143 occurs in the first 10 ms of the contraction. It is not till 40 ms after stimulation that the subsequent contraction compensates this increase. The maximum shortening which is only slight in proportion to rest occurs about 100 ms after stimulation.

Dynamic shortening is highest with *small degrees of stretch* where the maximum is reached more quickly. *With moderate or high elongations* the maximal value of shortening is reached later and the contribution to contraction tension given by changes in equilibrium length decreases and is negative in the first 20 ms of contraction. The negative contribution is of increasing importance with increasing elongation. When changes in equilibrium length contribute less to extra-tension, the part played by stiffness increases correspondingly. This is not surprising as a certain degree of stretch is necessary for extra-tension to arise from increase in stiffness. The fibre has a shortening tendency towards the same equilibrium length both before and after the increase in stiffness, the only difference being that the force with which the fibre recoils to its equilibrium length is correspondingly greater when stiffness increases.

2. The mechanical reactions of the fibre at different strengths of stimulation and different degrees of stretch.

The time course of *extra-tension* (Fig. 9), $\frac{\text{stiffness}}{\text{tension}}$ during contraction (F) compared to that in the resting fibre $\left(\frac{F}{F_0}\right)$ (Fig. 10), and of the *dynamic shortening* (Fig. 11) at different strengths of stimulation is shown in a three-dimensional coordinate system, where the abscissa is the time in ms, the ordinate the changes caused by contraction and the z-axis the degree of stretch.

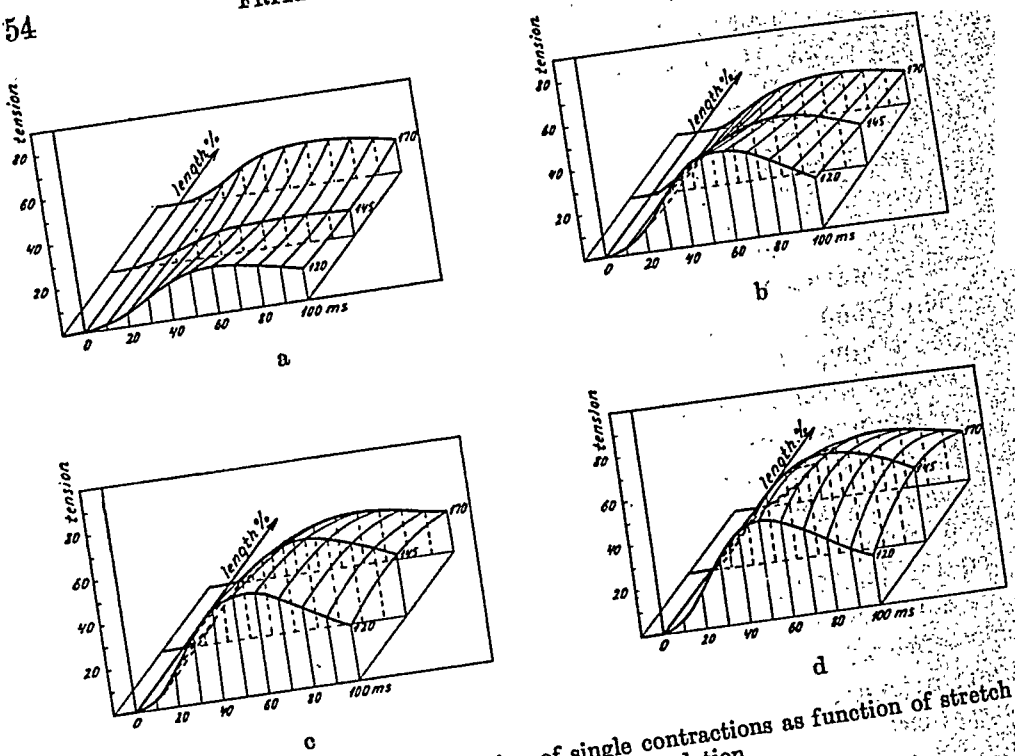

Fig. 9. Time course of extra-tension of single contractions as function of stretch and strength of stimulation.

a) 1 × threshold stimulus
b) 2 × » »
c) 4 × » »
d) 10 × » »
Ordinate I: tension in arbitrary units
II: length in per cent of equilibrium length.
Abscissa: time in ms.

Gradation of extra-tension by using varying strengths of stimulation (Fig. 9 a, b, c and d) is highest at lengths 120 and 145 just as extra-tension proper is highest here. The time course of the contraction tension is uniform with different strengths of stimulation and the tension maximum is reached later with increasing degree of stretch. Regarded as function of stretch extra-tension varies irregularly with threshold stimulation. When the strength of stimulation is 2 or 4 times greater, extra-tension falls with increasing length. When it is 10 times the treshold value extra-tension is constant at low or moderate degrees of stretch but falls with high elongations.

$\frac{\textit{Stiffness}}{\textit{tension}}$ *during contraction* compared with that at rest $(\frac{F}{F_0}$

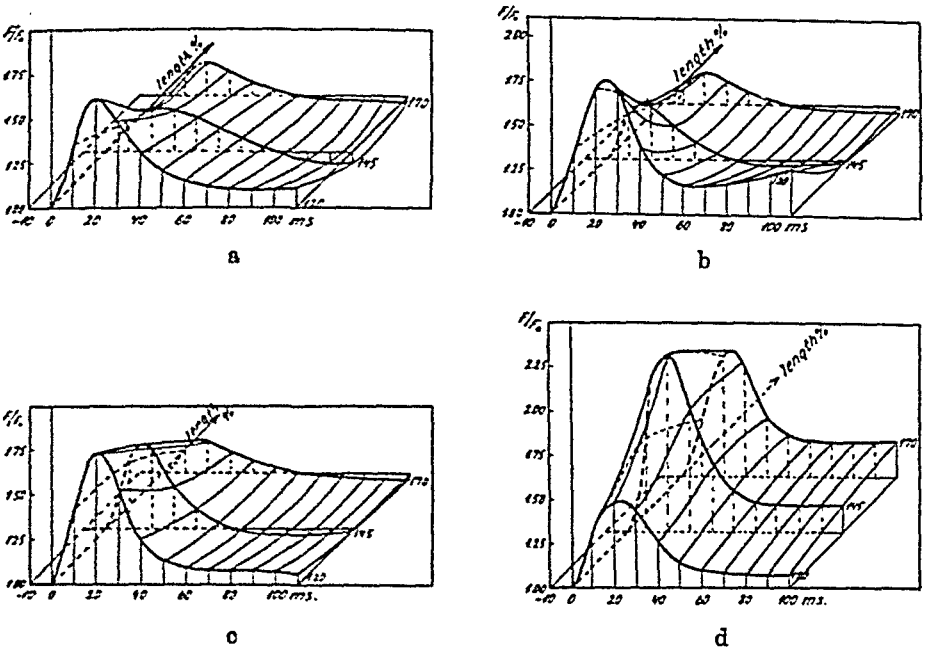

Fig. 10. Time course of F/Fo during single contractions as function of stretch and strength of stimulation.

a) 1 × threshold stimulus
b) 2 × » »
c) 4 × » »
d) 10 × » »
Ordinate I: F/Fo
II: length in per cent of equilibrium length (equilibrium length = 100)
Abscissa: time in ms.

Fig. 10 a, b, c and d) at length 120 has lower values when stimulation is strong than when it is weak. At lengths 145 and 170 $\frac{F}{F_o}$ increases with increasing strength of stimulation. The time course of the coefficient varies somewhat for different strengths of stimulation, and no systematic changes other than those at maximal contraction described in the foregoing section (see page 51) are found. In the last phase of contraction $\frac{F}{F_o}$ at moderate or maximal stretch and moderate strengths of stimulation is below 1, i. e. the fibre in this phase of contraction is less stiff than when at rest. With increasing degree of stretch $\frac{F}{F_o}$ decreases when the strength of stimulation is 1—4 times the threshold value. When

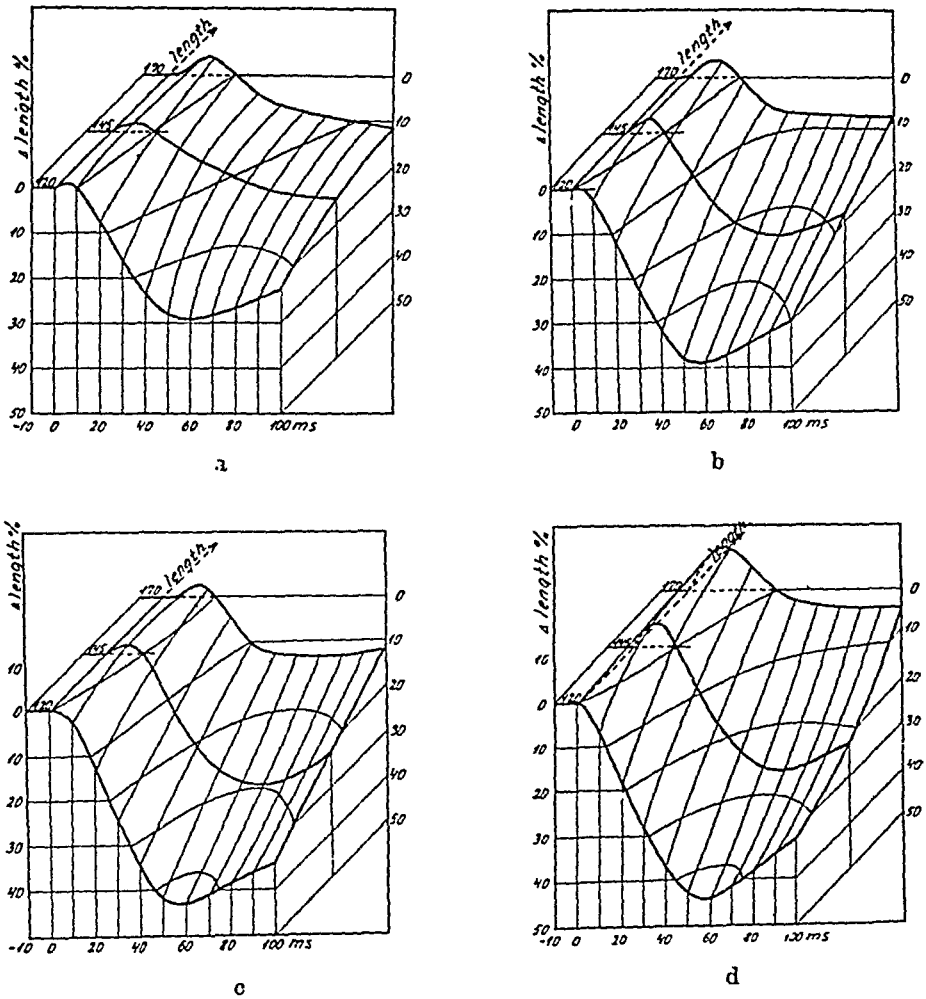

Fig. 11. Time course of dynamic shortening (Δ length) during single contraction as function of stretch and strength of stimulation.

a) 1 × threshold stimulus.
b) 2 × » »
c) 4 × » »
d) 10 × » »

Ordinate I: Δ length (cf. equation (21) pg. 52).
II: length in per cent of equilibrium length (equilibrium length = 100).

Abscissa: time in ms.

it is 10 times the treshold value, a maximum for the coefficient is found at moderate stretch (Fig. 10 d).

The time course of the *dynamic shortening* with different strengths of stimulation and degrees of stretch is given in Fig. 11 a, b, c and d. At length 120 shortening increases evenly with increasing strength of stimulation. At length 145 an elongation

occurs in the first phase of the contraction which increases with increasing strength of stimulation. The following shortening also increases with stimulation strength but is less than at length 120. At threshold stimuli the time course deviates somewhat from the course at high stimulation strength, as the maximal value in shortening is not attained until 100 ms after the moment of stimulation. At length 170, just as at the beginning of contraction in the moderately stretched fibre, an essential increase in dynamic equilibrium length takes place, which is most marked when stimulation is maximal. The following shortening is lowest at maximal stimulation and highest when stimulation is 4 times the threshold value. *Thus the shortening tendency decreases with increasing stretch at all strengths of stimuli.*

When determining *the static equilibrium length in tetanic contraction at different strengths of stimulation*, only a slight change in the shortening ability is found with increasing strength of stimuli (in contrast to the dynamic experiments just described). When rising strength of stimulation causes a tension increase in the ratio 1 : 3, the shortening ability varies only 15 per cent at low and 25 per cent at medium degrees of stretch. A comparison with changes in equilibrium length at different stimuli in dynamic experiments shows that here shortening following maximal stimuli varies twice as much as in the static determinations. In static experiments, as will be accounted for later, one would expect a higher gradation in equilibrium length. Phenomena in connection with yielding and locking are probably the cause of this higher gradation being masked (see page 72).

3. *Temperature dependence of mechanical reactions.*

The previous experiments concerning the influence of stretch and stimulation on the mechanical reactions of the fibre are carried out at a temperature of 19° C. When analysing the factors determining size and course of mechanical reactions, a knowledge of their temperature dependence may help to elucidate structural properties. *Temperature dependence of tension and stiffness in the resting fibre* is treated in the preceding investigation. The present material concerns only determination of dynamic stiffness and shortening.

$\frac{\textit{Stiffness}}{\textit{tension}}$ *at rest.* In the range of temperature examined (3—25°) the same stiffness and stiffness-tension dependence is found within the limits of accuracy. At 25° C there is a sudden increase in stiffness corresponding to an irreversible heat contracture. It is

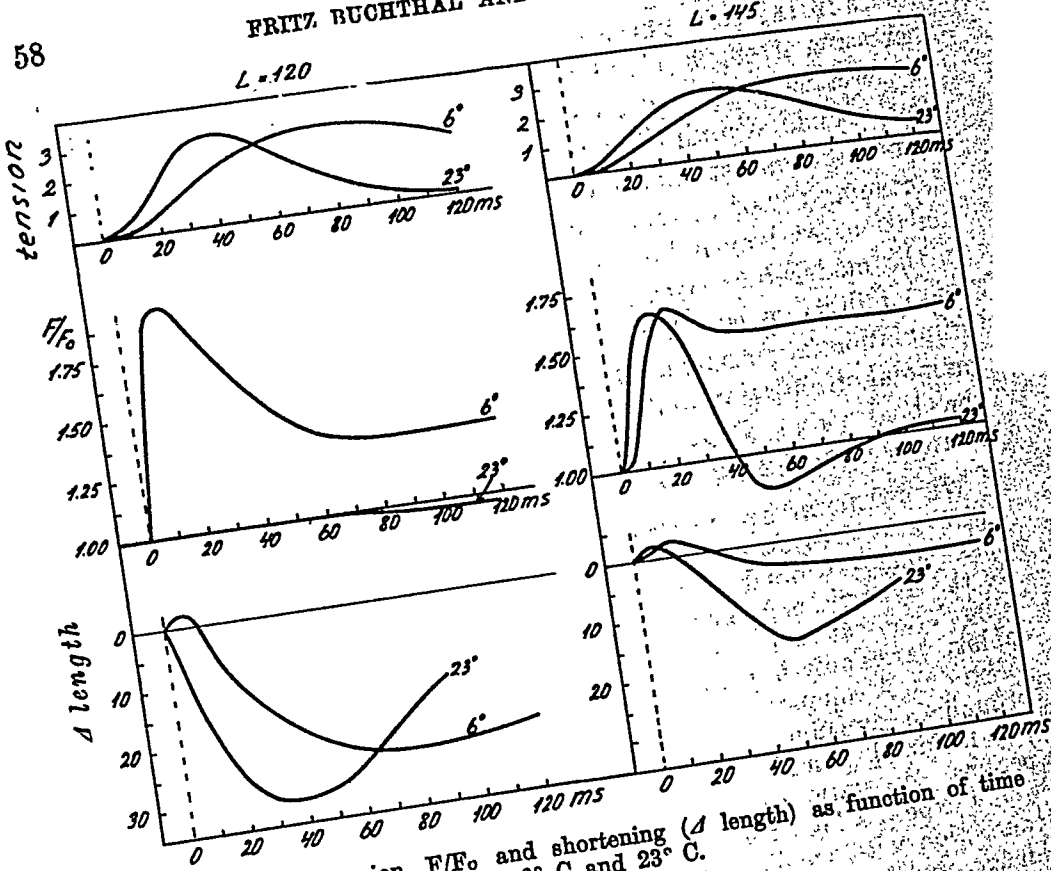

Fig. 12. Extra-tension, F/F_0 and shortening (Δ length) as function of time at 6° C and 23° C.

Left curves: L = 120
Right curves: L = 145
(equilibrium length, L = 100)

Ordinate: (top) tension in arbitrary units; (middle) $\frac{F}{F_0}$; (lowest) Δ length (cf. equation (21) pg. 52).

surprising that stiffness resulting from elastic and viscous elements is not affected by temperature in the resting fibre when noticeable viscous forces which should depend on temperature must here be reckoned with.

Height of extra-tension during contraction (Fig. 12 top) is only slightly affected by changes in temperature (6—23° C), while the time course of contraction becomes essentially delayed at lower temperatures. Extra-tension decreases below 6° C. In tetanic contraction the size of tension is affected even above 6° C.

Stiffness in single contraction (Fig. 12, middle). The time course of $\frac{F}{F_0}$ during single contraction is strongly affected by

temperature. At low elongations (length 120) $\frac{F}{F_o}$ remains unaltered during contraction initiated at 23° C, at 19° C it varies slightly (Fig. 8 b), and at 6° C the change in $\frac{F}{F_o}$ during contraction is considerable. At moderate and high degrees of stretch $\frac{F}{F_o}$ increases at high temperature about 50 per cent in the initial period of contraction. At low temperature it rises suddenly 50 per cent and stays at this value throughout the contraction.

The dynamic shortening (Fig. 12, lowest) caused by single contraction varies only slightly with changes in temperature at *small degrees of stretch.* It is, however, worthy of note, that here at low temperature there is an increase in equilibrium length in the initial phase of contraction.

At moderate and high degrees of stretch, temperature has a great influence on dynamic shortening which shortening is less at low than at high temperature.

To summarise, the experiments on temperature dependence show that tension in the resting fibre rises with increasing temperature (3—25°C, BUCHTHAL et al. 1944) while extra-tension in single contraction is only slightly affected. *Dynamic stiffness* at rest is not influenced by changes in temperature but during contraction falling temperature causes an increase in stiffness. Dynamic shortening decreases at moderate and high degrees of stretch with falling temperature.

4. Mechanical reactions of the fibre during fatigue and after restitution.

Extra-tension, stiffness and dynamic shortening are recorded during different degrees of fatigue, brought about by inserting tetanic contractions of different durations. The example in Fig. 13 shows extra-tension and $\frac{F}{F_o}$ in the non-fatigued fibre (curve I) while curves II—IV give values for the progressive degrees of fatigue. The decreasing tension due to fatigue is accompanied by decreasing $\frac{F}{F_o}$ in the initial phase of the contraction. On the

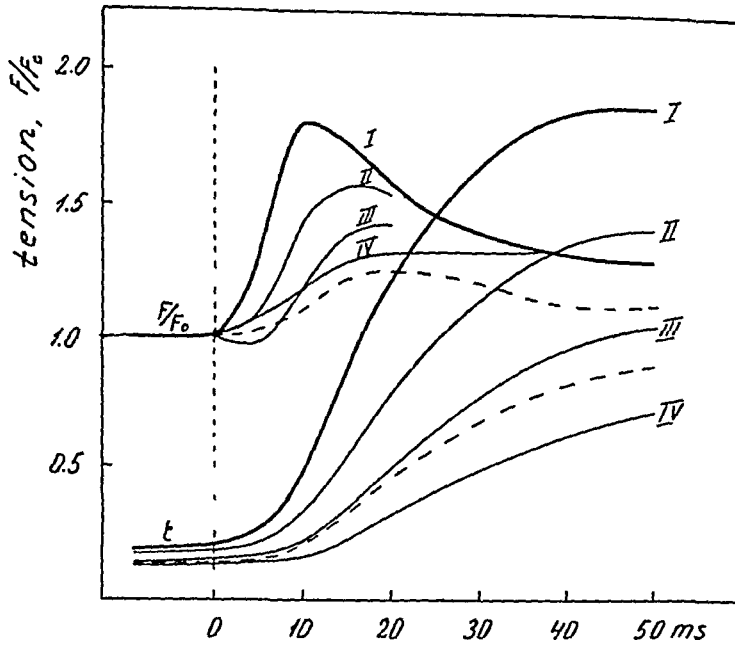

Fig. 13. Extra-tension (t) and F/Fo as function of time at different degrees of fatigue.

I non fatigued fibre.
II—IV progressive degrees of fatigue.
- - - - - partial restitution.
Ordinate: tension in arbitrary units and F/Fo
Abscissa: time in ms.

other hand $\frac{F}{F_o}$ is not affected by fatigue when extra-tension is at its maximum. After a few minutes rest, despite increasing initial and maximal tension, $\frac{F}{F_o}$ lies considerably lower than in the fatigued and non-fatigued muscle fibre. Increase in extra-tension after partial restitution is thus due to a higher dynamic shortening. Dynamic shortening is most considerable in the non-fatigued and recovering fibre, while different degrees of fatigue have a lowered and somewhat constant dynamic shortening.

In previous investigations of gradation in cross striation $\left(\frac{A}{A+1}\right)$ (BUCHTHAL and KNAPPEIS 1943), a considerable change in $\frac{A}{A+1}$ in contraction is found when comparing the fatigued with the non-fatigued fibre. Without fatigue, only in the strongly stretched fibre does a change occur in cross striation corresponding to gradation in contraction tension. Lack or presence of gradation in cross striation res-

pectively, is explained by the interaction of contracted and shunting substance at rest. At medium degrees of stretch the changes in this relation have no measurable influence on $\frac{A}{A+1}$. The change in cross striation caused by fatigue just observed at moderate elongations where contraction tension does not decrease more than during variation of stimulation, is not explained by a decrease in the proportion between contracted and shunting substance at rest. *The only possibility left is that the change in* $\frac{A}{A+1}$ *caused by fatigue is due to variations in equilibrium length localised in the single contracting elements themselves.*

5. The influence of frequency of the periodic changes in length on the mechanical reactions of the fibre.

The influence of the measuring frequency for determination of stiffness at rest and during tetanic contraction is discussed in detail in the preceding communication. In connection with the present work, only a series of observations will be mentioned where stiffness-tension diagrams at rest and F during rest and contraction are recorded at frequencies which exceed 100 cycles per sec. Stiffness-tension (b see page 41) increases with increasing frequency. This agrees with the fact that stiffness is more dependent on frequency at low than at high initial tensions of the fibre. The variations in F caused by single twitches have the same course in the frequency range 100—200 cycles per sec but the maximal change falls with rising frequency. This is in accordance with the previous experiments where the difference between rest and tetanic contraction decreases with increasing measuring frequency.

6. Extra-tension and stiffness in the initial phase of contraction.

Throughout the experiments described so far, proportionality was found between stiffness and tension at rest and during contraction. The proportionality factor F_0 is independent of tension and temperature at rest. F has a higher value during contraction than when at rest. *At the start of contraction F increases very rapidly within a period where tension develops slowly, i. e. development of stiffness occurs more rapidly than that of tension.*

Fig. 14 shows the initial course of extra-tension, stiffness and coefficient $\frac{\text{stiffness}}{\text{tension}}$ (F) in single contraction. Tension develops

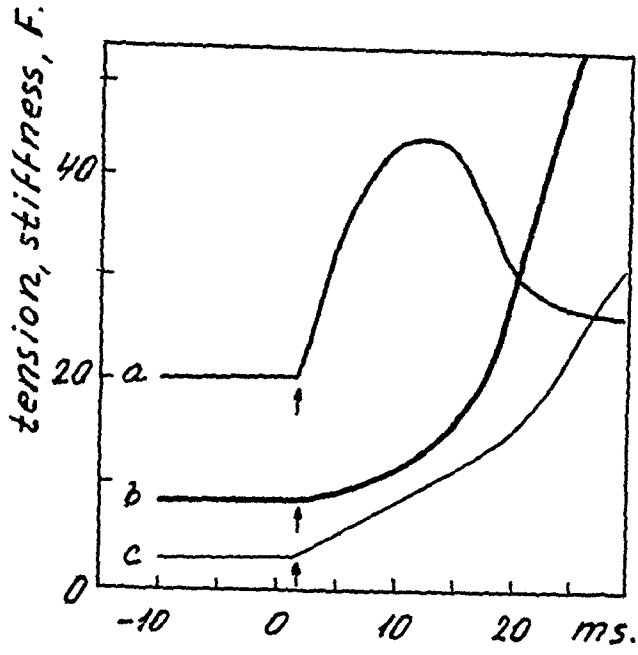

Fig. 14. Initial course of extra-tension (b) stiffness (c) and F (a) during single contraction as function of time (ms). Stimulation at 0.
The arrow marks the place where the first mechanical reaction can be traced.

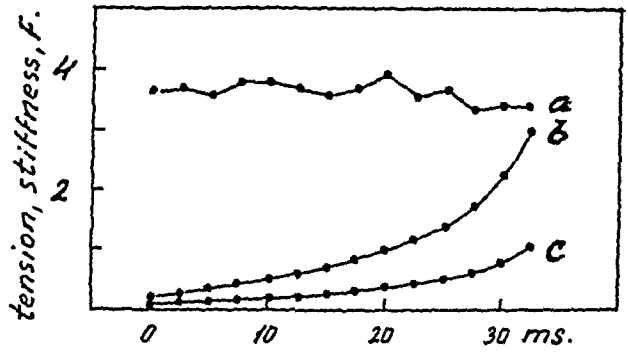

Fig. 15. Rapid elongation of resting fibre (mean curve of 4 experiments). Tension (b) stiffness (c) and F (a) as function of time.
Ordinate: tension, stiffness and F in arbitrary units.
Abscissa: time in ms.

the first 10—15 ms with an evenly rising velocity viz. its course is curved, while stiffness increases linearly with a marked inversion point 1—2 ms after stimulation. Therefore, F first rises steeply and attains a maximum in the course of 12 ms after which it decreases and remains constant (above rest value) during the main part of contraction.

In order to investigate whether the different initial development of stiffness and tension is inherent in the contraction process proper, or whether it is caused by the sudden increase in tension, tension and dynamic stiffness in the curarised resting fibre are recorded during rapid extension to reproduce as well as possible the time course of the tension at the start of the single twitch. The curves (Fig. 15) show the characteristic properties of the resting fibre. F is constant, independent of the rate of tension, and stiffness changes proportionally with tension. *The difference in rate of development of stiffness and tension must, therefore, be regarded as resulting from the contraction process itself.*

The different initial course of the curves is due to 1) the difference in rate of the relative increase in stiffness and tension in the single fibre elements at the start of contraction and 2) the propagation time. At smaller fibre lengths the curved period of tension development decreases and stiffness increases more steeply. (transition period shorter). The tension would probably develop linearly and stiffness rise instantaneously were the part of the fibre so short that propagation time could be ignored. Working on this assumption for a single fibre element, we shall construct the resulting curves of stiffness and dynamic shortening for a total fibre. While the contraction propagates over the fibre, stiffness suddenly increases strongly in the single fibre elements whereafter it decreases gradually to the contraction value proper. By supposing that transition from maximal stiffnesss to a more or less constant contraction stiffness is about half the propagation time we get, for the *total fibre*, resulting curves of F and dynamic shortening as shown in Fig. 16, curves a and b. The supposed variations of F and the dynamic shortening for a *single fibre element* are seen in curves a_1, and b_1, The curves constructed for the total fibre (a and b) agree with the course of F and dynamic shortening found experimentally.

At a superficial glance the different rate of development for stiffness and tension together with a finite rate of propagation manifest themselves by a time difference between the start of stiffness and tension during contraction. *Within a period where extra-tension is very slight, often so slight that there is some doubt as to whether the latent period is finished or not, a considerable change occurs in F*, which is inherent in the contraction process proper. This disproportionality is also found in the initial phase of tetanic contraction. (Fig. 6).

It is of interest to investigate in how far factors influencing contraction affect the difference in rate of development of stiffness and tension.

Influence of stretch. As long as the stiffness of minute structure is masked by the coarse micellar adjustment as is the case at equilibrium length and lengths below 100, the rapid increase in

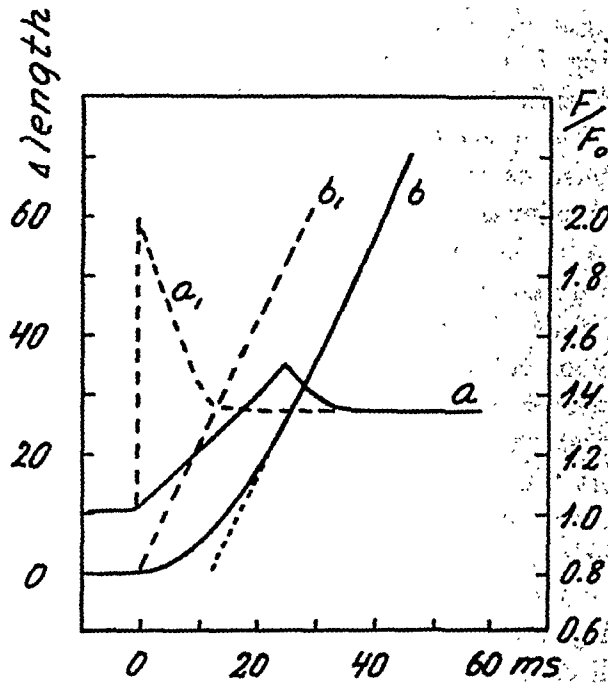

Fig. 16. F/Fo (a) and shortening (b) at start of contraction for one fibre element (a_1 and b_1) and for the total fibre (a and b).
Ordinate: F/Fo and shortening (Δ length) in arbitrary units.
Abscissa: time in ms.

stiffness cannot be observed, even if it is presumed to be present. At small or medium degrees of stretch conditions are present for the reflection of minute structure in the resulting stiffness and the difference in rate of development becomes marked. The difference disappears again with high degrees of stretch, because the original stiffness is large and the effect of the increase in stiffness in the initial phase of contraction becomes of less importance.

The rate in stiffness and tension development is affected not only by the degree of stretch but also by the *absolute length of the fibre.* For stiffness as well as tension the rate in development increases with *decreasing length* as the propagation time is shortened and the time course of the curves approaches that supposed for the single fibre element. (Fig. 16 a_1 and b_1).

A variation of the *strength of stimulation* likewise effects a change in the mutual relation of rates of development. At threshold value determination is difficult because of the small size of mechanical reactions in the initial phase of contraction. When the stimulus is 1,5—2 times the threshold value, there is a greater difference between the rates of development for stiffness and tension, which decreases with increasing stimulation strengths. The structural interpretation of this dependence is discussed in a following section (see page 71). An investigation of the *temperature dependence* of the rates of development shows a change proportional with the latent period which shortens 10—12 per cent per degree rising temperature.

The following is a short review of observations on the mechanical reactions of the fibre found previously and in the present investigation, which must be contained in a structural interpretation.

The basis for an estimation of the mechanical properties is a determination of the *mutual relation between length, tension and stiffness* under different experimental conditions.

Length-tension diagrams at rest and during isometric contraction are discussed in a former investigation (BUCHTHAL 1942). In connection with an analysis of minute structure we may mention that an *irreversible change in length of the fibre* (*yielding*) occurs when contraction tension has reached a certain level, after which the fibre is elastically locked in the new length as long as contraction lasts. This fact is revealed by release-length-tension diagrams, among others, which during contraction lie appreciably lower than the corresponding extension diagrams.

A proportionality between stiffness and tension is characteristic of the elastic properties of the fibre. The proportionality factor increases differently at rest and during contraction when the frequency of the periodic changes in length used for measuring stiffness is varied. *This frequency dependence* indicates the presence of viscosity in the structure. Its influence is greater during isometric contraction than at rest and greatest during release contraction.

When measuring contraction stiffness at low frequencies, yielding causes irregularities in the otherwise linear dependence between stiffness and tension. At these frequencies stiffness-tension diagrams have a critical point (maximum). *At higher frequencies contraction-stiffness is proportional with tension and the*

proportionality factor is greater during contraction than at rest. The difference between rest and contraction decreases with frequencies above 100 cycles per sec.

The proportionality factor is greatest during tetanic contraction initiated at *small degrees of* stretch and decreases if the contracting fibre is subjected to higher tension (yielding). Yielding is considerable in the first part of a tetanic contraction as is seen from the low initial stiffness present despite constant tension. At higher degrees of stretch, where contraction extra-tension disappears (*indifference point*), *extra-stiffness decreases in a corresponding way.* In contrast to tetanic contraction the proportionality factor in single contractions does not decrease evenly with stretch, but has a maximum at moderate elongations.

When increasing strength of direct stimuli we find an increase in tension and stiffness. In connection with this gradation of tension and stiffness following changes in strength of stimulation, it is interesting to note that the proportion between lengths of the isotropic and anisotropic substance $\left(\frac{A}{A+I}\right)$ remains constant at small and moderate degrees of stretch. Changes in $\frac{A}{A+I}$ only manifest themselves at high degrees of stretch.

There is a considerable influence of temperature on contraction stiffness as the fibre becomes stiffer with falling temperature.

From *tension and stiffness* the corresponding *dynamic shortening* is computed, which is highest at small degrees of stretch and decreases with increasing stretch and falling temperature. When conditions are such that dynamic shortening decreases during contraction, it decreases still further with increasing strength of stimulation.

Proportionality between stiffness and tension is lacking *in the initial phase of single twitches and tetanic contraction* as an increased stiffness occurs in this phase which results in a lowered shortening ability. The shorter the fibre, the more rapid is the development of stiffness and tension (progagation time).

Minute structural interpretation.

The contractile elements are regarded as consisting of long molecule chains with an odd number of charge series which are found in the resting fibre in a quasi-stationary equilibrium state

(BUCHTHAL 1942). Variations in length arise here as a result of angular movements in the single links. This observation is supported by the linear course of stiffness-tension diagrams. A change in potential at any of these charge points (corresponding to a "stimulation quantum") will cause a chain reaction, so that the alteration in charge is transmitted, and when propagated, will cause a decrease in distance between the single links. *This decrease in distance corresponds to the decrease in equilibrium length occurring during contraction.* When the length of the total chain is kept constant (isometric) the links still at rest will be stretched and a *mechanical tension* will arise. Besides a change in equilibrium length we find an increase in stiffness due partly to tension (1) partly to the contraction process proper (2).

1) Increase in stiffness due to tension is caused by the orientation of the substance — its molecule chains and their single links. With increasing tension, length is increased and with it orientation which is closely connected to changes in equilibrium length. The higher the orientation the greater the force needed to induce a further straightening out. This increase in stiffness due to tension is a characteristic property of *all* highly elastic materials and is independent of the contraction process as such.

2) The contraction process itself causes an increase in stiffness which, however, at low measuring frequencies is masked by yielding localised in the contracted elements of the molecule chain. This increased stiffness is probably due to larger forces which arise when the distance between the single links decreases.

Looked at superficially these two causes of the change in stiffness explain most of the experimental observations.Thus increase in stiffness during contraction is mainly due to the larger stiffness of the contracted links. The higher the original length the less is this increase, as the number of contracted links in the single molecule chain decreases with stretch. The same phenomenon manifests itself by a decrease in stiffness when a contracted fibre is strongly elongated and thereby a number of the contracted links are straightened out to a resting condition (yielding). *The elongation at rest* determines how large a part of the contracting chain participates in contraction. At high degrees of stretch the distances between the single links become so great that the contraction cannot propagate, an extension which corresponds to the indifference point of the length-tension diagram.

In tetanic contraction the *transition link* between the contracted

and resting parts of a chain from which the stimulation quantum jumps to a neighbouring chain, offers possibility for an explanation of the *elastic locking* evident in release contractions. When releasing during contraction, the mechanical pre-requisites for further propagation of contraction over the resting links are established only when the formerly stretched links have consolidated in a shorter length. Until this happens, the stimulation quantum will stop at the *same* transition link and the fibre appears elastically locked. The same interpretation is valid for single contractions but here maximal increase in stiffness is not attained at low degrees of stretch, as the extra-tension is insufficient to complete the coarse structural adjustment necessary when stiffness measured is to express minute structural stiffness.

Experiments for determining elastic after-effects and dependence of stiffness on frequency and temperature indicate that, besides the purely elastic, a viscous component is found in the mechanical system which cannot be explained by assuming purely elastic elements during rest and contraction. There are two main possibilities which may explain these phenomena.

1. Intrinsic friction and 2. modifications of the substance caused by stretch and tension respectively (changes in linkage). There is, of course, a certain amount of friction, but probably this is quantitatively of minor importance. Intrinsic friction cannot explain in a simple way why viscosity increases with stretch at rest, while it decreases during contraction. Furthermore, it cannot explain why viscosity varies strongly with temperature during contraction but hardly at all at rest. There is, therefore, reason to suppose that, as far as contraction is concerned, the essential viscous factors are due to changes in linkage so that in any case there are two modifications dependent on stretch.[1] Given the mechanical conditions for the new modifications a transition can only be initiated by accidental effects e. g. warmth movements (H. H. Weber 1934, 1941). At higher elongations there are linkages (looser or longer) which cause greater equilibrium lengths. The fibre elements will, therefore, appear strongly viscous, as transition from a long modification at high stretch to a shorter at low stretch cannot take place instantaneously but requires a certain time. With rapid increase in length, tension will,

[1] For rubber, X-ray spectographic experiments have shown the appearance of a new modification at higher elongations (R. W. Asmussen cit. Buchthal 1942).

therefore, fall quickly only to rise again when the new modification is established causing a shorter equilibrium length and higher tension. Thus, a given rapid change in length effects a higher tension difference than a slow change. *Stiffness is, therefore, dependent on frequency. The difference between static and dynamic stiffness is caused by the relation between the rate of changes in length and the rate with which the new modifications adjust themselves.*

In a mechanical equivalent the purely elastic stiffness and that due to changes in linkage are placed in series (GASSER and HILL (1924), LEWIN and WYMAN (1927), BUCHTHAL (1942). If the latter stiffness is blocked by inhibiting the changes in linkages (low temperature or work in a range of stretch where not more than one linkage state is possible), only purely elastic stiffness is left. The sudden increase in stiffness which occurs at maximal stimulation during transition from low to medium degrees of stretch (Fig. 10 d) may be due to the fact that here there is only possibility for one, while at low elongations there are two or more modifications between which transition is relatively easy.

Hitherto the mechanical properties of the fibre have been deduced from properties of one minute structure element (one molecule thread). For a further understanding of experimental observations in connection with propagation of stimulation and the reaction of the fibre at different stretch and stimulation, it is necessary to consider *the mutual interaction of neighbouring elements.*

When stimulation is above threshold at any point of the fibre a number of stimulation quanta increasing with increasing strength of stimulation are released, which in turn initiate contraction and jump from molecule thread to molecule thread. When contraction no longer propagates over the chain the stimulation quantum jumps to a neighbouring chain. Probably the quanta move at random over the fibre, so we can have stimulation quanta moving backwards and forwards. This assumption is supported by the fact that locally released contractions are transmitted in both directions. Furthermore it must be supposed that, little by little, the stimulation quanta become neutralised. If this were not the case, a twitch would become a lasting contraction as the stimulation quanta would jump backwards and forwards infinitively between the molecule threads making them contract. On the other

hand contraction propagates over the fibre without decrement with regard to both mechanical reaction and microscopic structure. The decrease in stimulation quanta for propagation over the total fibre must, therefore, be less than half their number, as halving the strength of maximal stimuli gives just noticeable changes in mechanical reaction. The rate of relaxation is probably dependent on processes during transition from contraction to rest as well as the activity of stimulation quanta still present. The maximal tension developed in a *single twitch* is determined by the amount of stimulation quanta released in a given time. During *tetanic contraction* a greater number of chains must be brought into simultaneous contraction to produce higher tension (3—4 times that developed in twitch).

There is no reason to suppose that release of stimulation quanta at the stimulation point requires a measurable time for development and the finite *latent period* is apparently in contradiction to this interpretation. It must, however, be remembered that the stimulation quanta released in the moment of stimulation can only produce a *purely local contraction at the stimulation point*, an effect so slight that even with highly sensitive recording technique, it can only be observed when the contraction has propagated over the greater part of the fibre. This interpretation is supported by experiments *comparing latent period in long and short fibres*. Only with a high measuring sensitivity can we find the same short latent period in both. At 19° C about 2.0 ms elapse before measurable tension occurs, but there is no reason to suppose that tension development does not occur instantaneously as does development of local potentials in nerve. The rapidly increasing stiffness likewise indicates that mechanical processes in the fibre manifest themselves very soon after the moment of stimulation and this makes a finite latent period for the directly stimulated muscle fibre very problematic.

From stiffness of the resting and contracting substance and gradation in cross striation due to changes in strength of stimulation at low and high elongations, it follows that contraction does not include all molecule threads in a micella, and that more or less resting substance shunts the contracting substance, all depending on the strength of stimulation. For the degree of stretch concerned the minute structure elements have an "all or none" reaction (BUCHTHAL and KNAPPEIS 1943). Gradation of tension, when strength of stimulation is varied, is brought about

by a gradation of the number of contracting elements. This interaction of contracting and shunting resting substance causes *changes in intrinsic tension.* Thus, apart from a shortening tendency an intrinsic tension arises during contraction, which causes an increase in stiffness independent of the extrinsic tension.

This increase in stiffness will be especially marked in the initial phase of the contraction before a mechanical consolidation between contracting and resting substance is established. It is indicated by the more rapid development of stiffness compared with tension. It is most marked at medium strength of stimulation and moderate elongations. The structural changes are naturally slight at weak stimuli and it is therefore difficult to find any increase in stiffness. The intrinsic tension is most considerable at medium but decreases at maximal stimuli as the amount of shunting resting substance diminishes due to stronger contraction.

According to former conceptions of the mechanical reactions of the fibre, the sole cause of contraction was a decrease in equilibrium length. It is surprising, therefore, to find that under certain conditions *the dynamic equilibrium length* can even be *greater* during contraction. Although changes in stiffness were known to occur during contraction, no attempt has been made to explain their quantitative influence on mechanical reactions.

With a given tension an increase in stiffness will cause a lesser shortening ability and tension arising from changes in equilibrium length will decrease.[1] On the other hand, a decrease in stiffness will cause an increased shortening ability and thus the contribution of the change in equilibrium length to the resulting tension is increased.

Considering the changes in stiffness and equilibrium length from a structural point of view we may summarise as follows:

1) *The stiffness of a chain element* is determined by the direction and size of the forces holding each other in balance (linkages).

2) The stiffness in *one molecule chain* is determined by the stiffness and degree of orientation in the single link which in turn is dependent on the tension in the chain.

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[1] From equation $y = (e^{Fx} - 1)\ b$ (13, pg. 48) follows $F^x = \log\left(\frac{y}{b} + 1\right)$. When tension y is kept constant the product F_x will also remain constant i.e. shortening ability and stiffness are in inverse proportion.

3) The stiffness in a *molecule aggregate* is determined by stiffness in the single chains which depends on the resulting *extrinsic* and *intrinsic* tensions.

The increase in stiffness caused by intrinsic structural tension can be so considerable in the initial phase of the contraction that it causes a decreased shortening tendency compared with rest as it inhibits shortening and thereby the changes in equilibrium length during contraction. In static as well as dynamic experiments contraction equilibrium length changes as function of strength of stimulation. With an extra-tension variation of 1 : 3 the dynamic equilibrium length varies about 30—50 per cent. These variations are explained by the inhibiting influence of the resting substance on shortening which decreases with increasing strength of stimulation. When applying calculations similar to those used in a former investigation (BUCHTHAL and KNAPPEIS 1943) the variation can also be explained quantitatively. The absolute variation in contraction equilibrium length due to different strengths of stimulation has the same value in static and dynamic experiments, but in the former the relative change in shortening is considerably less than in the latter. This, however, does not necessarily mean an altered influence of intrinsic tension under static conditions where $\frac{st_{contr.}}{st_{rest}}$ is about 6. As this is less than under dynamic conditions a still higher variation in equilibrium length could be expected. During contraction under static conditions, however, a yielding arises whereby the shortening tendency of the fibre becomes practically the same in a wide range of stretch. The shortening tendency and tension of the fibre increase with increasing strength of stimulation as the amount of shunting resting substance decreases continuously. An increasing tension will, however, cause a stronger yielding, which opposes or compensates the decrease in equilibrium length.

Thus, apart from extrinsic tension, stiffness is caused by properties of the links, of the chains and of the mutual interaction of different chains. During contraction stiffness increases because of a) increased stiffness in the contracting links b) intrinsic structural tension (inhomogeneous tension distribution) and c) increase in the tension itself.

Summary.

The mechanical factors determining tension in contraction of the single cross striated muscle fibre are investigated by a continuous registration of stiffness and tension at rest and during contraction. The method applied is in principle the same as that described in the preceding paper and stiffness is determined with a measuring frequency of 100 cycles per sec.

Dynamic stiffness varies proportionally with tension at rest and during contraction, the proportionality factor at the contraction maximum being from 30—90 per cent above that of the resting fibre. It depends on the initial length and decreases when the contracted fibre is subjected to higher tensions (yielding).

From tension, dynamic stiffness and their mutual interdependence the dynamic shortening ability can be computed, and *the influence of stiffness and equilibrium length on tension is investigated under different conditions.*

At all elongations the maximum in stiffness precedes that of tension. Changes in dynamic equilibrium length contribute relatively less to the initial stage of contraction than to the other phases and its influence on the resulting tension decreases with increasing stretch. At the beginning of contraction dynamic shortening may even attain *negative values*, this decrease being the more pronounced the higher the strength of stimulation and the degree of stretch.

When *increasing the strength of direct stimuli*, tension, stiffness and dynamic shortening increase, the latter varying more with grading than does static equilibrium length.

The contribution of stiffness to the resulting contraction tension increases considerably with falling *temperature* while that given by dynamic shortening correspondingly decreases.

The continuously falling tension during *fatigue* is accompanied by a gradual decrease in stiffness, while equilibrium length has a lowered but rather constant value in different stages of fatigue. Increase in extra-tension after partial *restitution* is due to a higher dynamic shortening, stiffness still being essentially lower than in the fatigued and the non-fatigued fibre. The falling contraction tension during fatigue is not caused by a decrease in number of active elements but is propably due to gradual changes in

equilibrium length occurring in the single minute structural elements proper.

Proportionality between stiffness and tension is lacking in the *initial phase of contraction* (10—20 ms), and within a period where extra-tension is slight, often so slight that it is difficult to decide whether tension is present or not, considerable changes occur in fiber stiffness which are inherent in the contraction process itself.

On the basis of the molecular model previously described a *structural interpretation* of mechanical factors determining tension is attempted.

Apart from extrinsic tension, stiffness is caused by properties of the molecular links and their modifications, by properties of the chains and by the mutual interaction of different chains. The initial difference in the developing rate of stiffness and tension is due to intrinsic tension caused by the interaction of contracting and shunting resting elements, before mechanical consolidation between the elements is established. This increase in stiffness can be so considerable that it inhibits shortening and causes a *negative* contribution of equilibrium length to the resulting tension.

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For a detailed review of the literature on this subject up to 1942 see Buchthal (1942).

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Is any Free Acetylcholine Preformed in Resting Muscles or in the Heart?[1]

By

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When the cholinergic nerve of an organ is stimulated, the "tissue acetylcholine" of this organ changes in some unknown way. Pharmacologically active acetylcholine is "liberated" and passes over into the perfusion or suspension fluid. Through many investigations following LOEWI and NAVRATIL's discovery of the choline esterase and the action of eserine (1926) we now know a great deal about the fate of acetylcholine after its liberation, but as to the origin of the liberated acetylcholine our knowledge is very scarce.

According to some authors the liberated acetylcholine is synthezised from choline, formed by hydrolytic breakdown of phosphatides and acetic acid deriving from the intermediary metabolism. The possibility of such a synthesis was made probable by LE HEUX (1921). He studied the pharmacology of a series of choline esters and found a special interest in the pyruvic and the acetic esters. Both of them were labile and both of them possessed a very great activity compared with free choline. On isolated gut LE HEUX found that addition of acetic acid or pyruvic acid specifically potentiated the action of added choline. The phenomenon was assumed to indicate that the gut synthetised the more active esters from the added choline and the organic acids in question. Later ABDERHALDEN and PAFFRATH (1925) and AMMON and KWIATOWSKI (1934) were able to show the synthesis of ach in

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enzymic systems. It was however necessary to use very large amounts of choline and acetic acid, and the yield of acetylcholine was only 1—2 pro mille of the theoretical value. Of late years the study of biological synthesis of acetylcholine has been forwarded chiefly by STEDMAN and STEDMAN and by QUASTEL and his collaborators. Studying the acetylcholine content of brain, STEDMAN and STEDMAN (1936, 1937) found that the yield of acetylcholine was considerably greater after the brain substance had been incubated at 37° C for some hours. Greater amounts of acetylcholine were also found after addition of chloroform. QUASTEL and his collaborators (1938, 1939) confirmed these findings. They also showed that the formation of extra acetylcholine was influenced by certain conditions. It was favoured by the presence of oxygen, and by the presence of glucose or certain intermediary products of carbohydrate metabolism. The formation of extra acetylcholine was assumed to be due to a synthesis. In these experiments it is, however, not possible to decide to what degree the formation of acetylcholine is due to a synthesis or to a breakdown of combined acetylcholine. The effect of oxygen, glucose etc. in the enzymic experiments of QUASTEL and co-workers and in experiments on perfused ganglion by MCINTOSH (1937) and KAHLSON and MCINTOSH (1937) strongly indicates that at least part of the acetylcholine formed is due to a synthesis.

QUASTEL and his collaborators also made another important observation (1938, 1939). When brain substance was exposed to certain agents the amount of pharmacologically active acetylcholine increased. The same finding had been made on other organs by some French authors (GAUTRELET, CORTEGGIANI, KASWIN and MENTZER, 1936; CORTEGGIANI, 1937; KAHANE and LEVY, 1936). This extra acetylcholine was assumed to be released from an inactive complex by the agents used (heat, acids, chloroform, acetone, alcohol). According to QUASTEL *et al.* the complex compound should consist of free acetylcholine and a protein molecule, which was supposed to be the ferment synthezising acetylcholine. The physiological rôle of the complex was only that of an intermediary stage at the synthesis of acetylcholine from choline and acetic acid. As to the co-ordination between nerve transmission and the biochemical processes, a synthesis of active acetylcholine via the complex compound of QUASTEL was supposed to occur at the very moment of excitation, (*vide* v. MURALT, 1939; BROCK, DRUCKREI, and HERKEN, 1939).

Other authors are not inclined to accept the proposed complex compound. STEDMAN and STEDMAN (1939) are of opinion that the formation of extra acetylcholine upon treatment of organs with various agents is due to a synthesis and not to a breakdown of a complex. They point out, however, that "the properties ascribed ... to their hypothetical complex are of such a nature that it is impossible directly to disprove its exsistence". TRETHEWIE (1938, 1939) also denies the presence of a complex. He stresses the fact that all experiments assumed to show the presence of a complex are made on whole organs or rough extracts containing cells. In his own experiments TRETHEWIE found a release of extra acetylcholine upon heating only in cell-holding extracts; if the extracts were centrifugated before exposure to heat etc. no acetylcholine appeared. TRETHEWIE concluded that the acetylcholine which appears when cell-holding material is heated does not derive from a complex but is ordinary acetylcholine which is protected from being extracted by the cell membranes as long as these are not destroyed by heat or chemicals.

Thus all authors seem to agree that acetylcholine is present in tissues and that this acetylcholine is synthetized from choline and acetic acid. Some authors have proposed the existence of a complex acetylcholine which should be an intermediary stage at the synthesis. We have looked upon this problem from a more pharmacological point of view and have come to the working hypothesis that active acetylcholine does not exist in resting tissues; all acetylcholine — not only a minor part of it — must be present as an inactive complex compound.

When acetylcholine is extracted and determined according to the methods hitherto used, the concentrations found in most organs amount to less than 1 γ (microgram) per gram of tissue weight. These amounts, however small in absolute figures, are surprisingly large considering the pharmacological activity of acetylcholine. In hearts the concentration of acetylcholine is found to be 0.1—0.3 γ per gram of tissue (*vide* GADDUM, 1936) but according to BEZNÁK (1934) 10^{-5} γ acetylcholine in 0.5 ml suspension fluid is enough to give a marked effect on the majority of 1000 frogs' hearts. As to ganglion, BROWN (1937) points out that the amount of free acetylcholine which can be extracted according to the usual methods is about 1000 times larger than the dosis which is sufficient to give an effect when administered by the "close arterial method". In other organs there have also been found

amounts of active acetylcholine which are considerably larger than the minimum effective concentration. This phenomenon may be explained in three ways:

1) in resting organs the pharmacological action of tissue acetylcholine is counterbalanced by an antagonistic mechanism,
2) within the cells the storage of tissue acetylcholine is — microtopographically — placed beyond the immediate neighbourhood of the receptors on which the acetylcholine molecules are supposed to act,
3) no free acetylcholine is present in resting organs. The acetylcholine found in extracts from resting organs is formed by a breakdown of a labile complex compound that is void of the pharmacological actions of free acetylcholine.

By assuming that tissue acetylcholine has other properties than free acetylcholine and changes into free acetylcholine upon stimulation or exposure to certain agents we may reasonably explain some phenomena earlier described in the literature. Thus we know from the studies of DALE and DUDLEY (1929) and CHANG and GADDUM (1933) on acetylcholine in horse spleen that the tissue acetylcholine must be present in a state that is not attacked by the esterase until after the organ is mechanically injured. To judge from the pharmacological action of eserine tissue acetylcholine is converted into an esterase labile state also after stimulation of cholinergic nerves. The tissue acetylcholine also differs from free acetylcholine in diffusibility. While added acetylcholine — to judge from its pharmacological actions — rapidly diffuses into an organ and is rapidly washed out, the tissue acetylcholine does not pass over into the perfusion fluid until the organ is stimulated. Mechanical injury is said to augment the amount of acetylcholine in the perfusion fluid (LORRENTE DE NÓ, 1938). These phenomena cannot be reasonably explained by alternative 1 or 2.

The discussion leads us to the working hypothesis that all acetylcholine in resting organs is present as an integral part of a complex which liberates active acetylcholine after stimulation, injury etc. If this theory holds true, the acetylcholine hitherto found with biological or chemical methods most probably derives from this complex, which is broken down during the course of analysis. From this working hypothesis follows the conclusion that the different methods used must have that quality in common which is the chemical condition for the splitting of the complex. This common feature seems to be the use of acid extraction fluids.

Most investigators emphasize the necessity of using acid extraction fluids to get the largest yield of acetylcholine from an organ. A 10 per cent trichloracetic acid is most commonly used. KAPFHAMMER and co-workers used 5 per cent metaphosphoric acid (1931). ENGELHARDT (1930) used n/10 HCl in 95 per cent ethyl alcohol. Several authors have, however, used eserinized salt solution for extraction. CHANG and GADDUM (1933) have thoroughly studied and compared the different methods. With HCl-alcohol for the extraction they found considerably smaller amounts of acetylcholine than with trichloracetic acid; with eserinized salt solution the yield was even smaller. It was believed that the more acid extraction fluid prevented hydrolysis of acetylcholine; ENGELHARDT'S HCl-alcohol mixture was not acid enough to prevent hydrolysis. This explanation is, however, contradicted by the fact that the stability of acetylcholine has its maximum at pH 3.9 (HOFMANN, 1934). On the alkaline as well as the acid side of this point the rate of hydrolysis increases (*vide* fig. 1). If 2 ml extraction fluid is used to every gram of tissue ("Muskelbrei") — the most common proportion — the acidity of the trichloracetic acid extract was found to be stronger than pH 1, the HCl-alcohol extract had a pH of 4—5, and the pH of the Ringer solution extract was 6—7. With regard to the stability of acetylcholine trichloracetic acid should be inferior to HCl-alcohol. It is necessary to look for another explanation of the fact that the yield of acetylcholine is greater when more acid extraction fluids are used. One possible explanation is that the very acid reaction caused by trichloracetic acid accelerates the liberation of free acetylcholine from the assumed complex. This view is further supported by the findings of BARSOUM (1935), who studied the efficacy of various extraction methods. He found greater amounts of acetylcholine with trichloracetic acid than with alcohol. Part of the "loss" in the alcohol extract was refound if the extract was treated with trichloracetic acid.

To support this discussion various organs were extracted with a fluid that gives a slightly acid reaction, *i. e.* 96 per cent ethyl alcohol cooled with solid carbon dioxide. As the breakdown of the eventual complex could be supposed to be accelerated by heat or by enzymes, the analysis were made on organs frozen with liquid air, solid carbon dioxide, or a mixture of ether and solid carbon dioxide. As far as possible all operations were made at low temperatures. The cell-free extracts obtained contained no free ace-

tylcholine although added acetylcholine was quantitatively refound. If the extracts were acidified and heated, amounts of acetylcholine appeared which were of the same order of magnitude as those found with the method of CHANG and GADDUM.

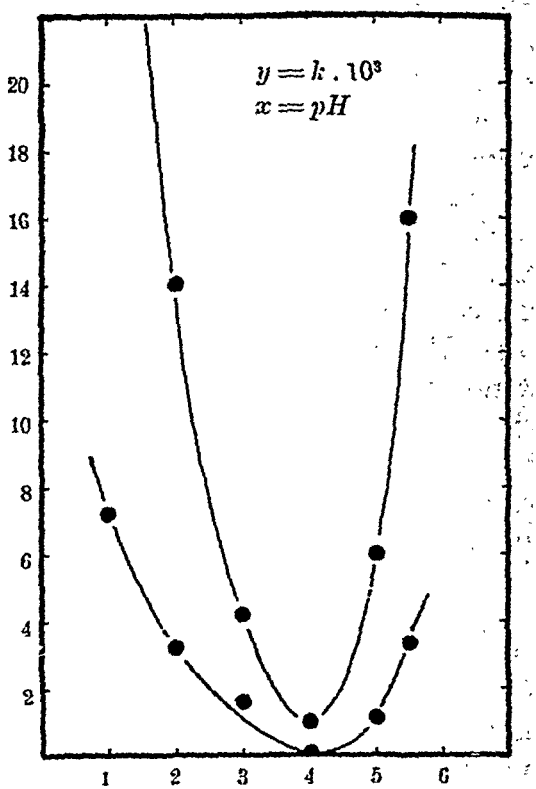

Fig. 1. (Rate of hydrolysis of acetylcholine at varying acidity).

In the experiments represented by the upper curve the concentration of acetylcholine chloride was 1 : 1,000,000, in those represented by the lower curve 1 : 200,000. Portions of these solutions were acidified through addition of HCl under the control of a glass electrode apparatus, transferred to glass bombs and heated on boiling water bath. After a measured period of time the glass bombs were cooled, their contents were neutralized and the remaining amounts of acetylcholine were determined on eserinized m. rectus of frog.

As we have found that the formula of monemolecular reactions can be applied on the hydrolysis of acetylcholine (ABDON and UVNÄS, 1937) the rate of hydrolysis is expressed as the constant, k, in this formula. (Brigg's logarithms were used at the calculation.)

Methods.

Most experiments were made on rabbit's hearts. For each analysis we used 3—5 hearts, each weighing about 4 grammes. The animal was shot through the head, and the heart was cut out. The blood was rapidly washed off and the still beating heart was embedded in carbon dioxide snow or thrown into liquid air or a mixture of ether and solid carbon

dioxide. The tissues were kept in Dewar flasks with solid carbon dioxide until worked up. As we must assume the presence of an enzymic mechanism for the breakdown of the complex all operations were made at temperatures below zero until the protein was denaturated.

The frozen organs were pressed to thin disks and then placed in a mortar containing 96 per cent ethyl alcohol. To every gram of tissue we used 2 ml of alcohol. The mortar and the alcohol had previously been cooled to about — 15° C. At this low temperature the disks softened in the alcohol so that it was possible to cut them into fine pieces with a pair of scissors and to grind them with quartz sand. The temperature was then allowed to rise to about 0° C and the mixture was stirred for 5 minutes. The extract was filtered through sintered glass filters with the aid of a suction pump. The residue was washed twice with 96 per cent alcohol, which was added to the extract. The extract was centrifugated 15 minutes at a rate of 3500 r. per minute. Such an extract is cell-free.

The extract has a slightly acid reaction. This is, however, caused by the carbonic acid which disappears during the following operations. The pH was therefore adjusted to 4.5 —6 with HCl (measured with "Lyphan paper"). The alcoholic extract was evaporated to about 1 ml at 30° C and at reduced pressure. As pointed out by ROMAN (1930), choline and its salts are volatile to a certain degree. The same thing seems to be the case with acetylcholine. When acetylcholine solutions are evaporated to absolute dryness the losses are considerable especially if the amounts of acetylcholine are very small. If they are evaporated to 0.5 or 1 ml. residue the losses are negligible.

To lessen the amount of disturbing impurities the residue was mixtured with its 20-fold amount of absolute ethyl alcohol insoluble matters were removed by centrifugation. It is often stated in the literature that the primary alcoholic extract of an organ (1 vol. tissue+2 vol. alcohol) should not contain any potassium salts. Potassium disturbs the biological assay of acetylcholine especially on leech preparations. Potassium is insoluble in 90—100 p.c. alcohol, but the water from the organs dilutes the extraction fluid to such an extent that practically all potassium of the organ is extracted. Therefore this re-dissolving in absolute alcohol was introduced. — The secondary alcoholic solution was diluted with about 40 per cent of water and evaporated in vacuo to about 1 ml. The addition of water was introduced when we found that acetylcholine disappears if a solution of it in 75—99.5 per cent alcohol is concentrated in vacuo. Possibly the alcohol is esterified by the acetyl radical (*vide* FOURNEAU, 1914).

The residue was washed twice with large amounts of ether. The ether was drawn off with a suction pump. The residue was rid of remaining ether by evaporation in vacuo. It was then dissolved in Ringer solution. The acidity was adjusted to pH 6.9.

The amounts of acetylcholine were determined by biological assay. As a rule we used *m. rectus abdominis* suspended in 2 ml Ringer solution with eserine sulphate 1 : 200,000. The addition of 0.02—0.05 γ was usually enough to produce a proper shortening of the muscle.

Results.

An extract of resting organs, prepared according to the method above, did not as a rule contain measurable amounts of free acetylcholine. In some few experiments we found small amounts of free acetylcholine, 5—10 per cent of those amounts, which are found with the usual methods. If the extracts were acidified and heated, large amounts of free acetylcholine appeared. It could be shown that this formation of acetylcholine was due to breakdown of a labile complex. We call this complex acetylcholine precursor.

a. Detection of a Labile Acetylcholine Complex.

Experiment 1 (acetylcholine precursor in rabbits' hearts).

5 rabbits' hearts, weighing 24.5 grammes, were extracted with alcohol according to the description above. The primary alcoholic extract was divided into 2 equal portions. One portion, called A-portion, was treated in full accordance with the description above. The other portion called B, was acidified with HCl until the concentration was 0.01 N and then heated to 80° C for 15 minutes. It was then cooled and the acidity was adjusted to pH 4.5—6. After that the B-portion was treated in accordance with and simultaneously with the A-portion.

Titration on eserinized leech preparations gave the following values:

A: no acetylcholine (i.e. less than 0.005 γ achCl per gram of tissue).
B: 0.21 γ achCl per gram of tissue.

So far the experiment shows that through the heating of the acidified alcoholic extract there has been formed a substance which caused the eserinized leech muscle to shorten. The precursor of the active substance is, however, present also in the A-portion; it has not been destroyed or lost at the operations made *viz.* evaporations, re-dissolving in absolute alcohol, washings with ether and the final dissolving in Ringer solution. When the non-active A-portion was acidified and heated, the active substance appeared in amounts which were of the same order of magnitude as in the B-portion, *i.e.* 0.185 γ acetylcholine per gram of tissue.

Experiment 2 (acetylcholine precursor in striped muscles of frog).

4 big ranae esculentae were anaesthetized with ether. Mm gastrocnemii were cut out and embedded in carbon dioxide snow. The weight of 8 muscles was 9.25 grammes. The A and B portions were prepared as in experiment 1. Titration on eserinized leech muscle gave the following values:

A: no acetylcholine (i.e. less than 0.01 γ achCl per gram of tissue).

B: 0.13 γ achCl per gram of tissue.

Experiment 3 (acetylcholine precursor in rabbit's brain).

A rabbit weighing 2.0 kilos, was anaesthetized with 3 grammes of ethylurethan given intravenously, and ether. The skull was trephined bilaterally. Preliminarily a thin bridge of bone was left in the median line to avoid injury of *sinus longitudinalis*. Great care was taken not to injure the brain or its vessels. When the whole brain had been laid bare the persisting bone bridge was rapidly cut off; the brain was lifted out on a spatula and thrown into liquid air. The performance of these last mentioned operations lasted less than 3 seconds.

Only the cerebral hemispheres, weighing 2.90 grammes, were used for the analysis. The A-portions was prepared as in experiment 1, and tested on eserinized mm recti abdominis of frog. A part of the A-portion was acidified with HCl to pH 5.5 under the control of a glass electrode apparatus, and then it was heated to 100° during 45 minutes. After being cooled and neutralized, it was tested on m. rectus of frog.

Before heating: 0.08 γ achCl per gram of tissue.

After heating: 0.48 γ achCl per gram of tissue.

Experiment 4 (Identification of the active substance formed).

In experiments 1—3 we found that extracts of slightly acid reaction prepared in cold contained practically no free acetylcholine. When the extracts were acidified and heated, an active substance was formed. Using the usual pharmacological tests we identified this substance as acetylcholine.

The extracts used for identification were prepared from frog's striped muscles. The extract was prepared and purified as described above and finally dissolved in Ringer solution. This is the A-portion. A part of the A-portion was acidified to pH 5.5 and heated to 80° C for 45 minutes. All experiments for identification were made on this extract.

The substance formed was found to have the following properties:

1. It had no effect on the non-eserinized leech muscle, but caused shortening after addition of eserine (fig. 2),
2. It causes shortening of the eserinized m. rectus abdominis of frog (fig. 3: 3).
3. On the frog's heart it produces negative inotropic and chronotropic effects, which were abolished by atropine.

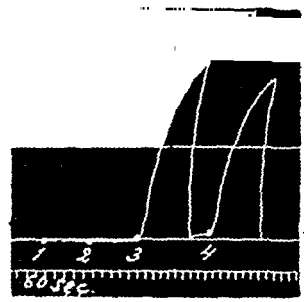

Fig. 2 (Identification of the active substance formed, see experiment 4).

Dorsal muscle of leech suspended in 2 ml Ringer solution. Between additions 2 and 3 the muscle strip was treated with eserine sulphate 1 : 200,000 for 60 minutes. At 1 and 3 addition of 0.01 γ of acetylcholine chloride; at 2 and 4 addition of extract, corresponding to 0.1 gram of tissue.

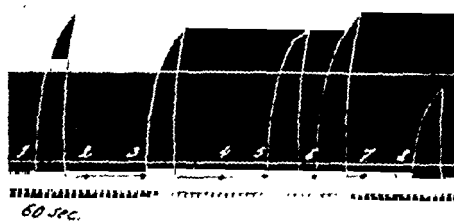

Fig. 3 (Identification of the active substance formed, see experiment 4).

M. rectus abdominis of R. temporaria suspended in 2 ml of Ringer solution with eserine sulphate 1 : 200,000. Between every addition of acetylcholine or unknown the kymographion was arrested for 10 minutes.

At 1 and 6 addition of 0.05 γ of acetylcholine chloride. At 2 addition of tissue extract corresponding to 0.5 gram of tissue before liberation of the active substance, ("A-portion"). At 3, 4, 5, 7, and 8 addition of the same amount of extract, which had been previously acidified and heated, "heated A-portion". At 3 "heated A-portion" without further treatment. At 4 "heated A-portion" exposed to non-eserinized blood serum. At 5 "heated A-portion" exposed to eserinized blood serum. At 7 "heated A-portion" exposed to 100° C after addition of NaOH. At 8 "heated A-portion" exposed to 100° C after addition of HCl to pH 5.

4. It looses its activity after being heated in alkaline solution (1 drop of 2-N NaOH to 2 ml extract, heated for 2 minutes, cooled, neutralized and tested, fig. 3: 7). It is relatively stable when heated in acid solution (after addition of HCl to pH 5.0 under the control of a glass electrode apparatus, the extract was heated to 100° C for 10 minutes, cooled, neutralized and tested), fig. 3: 8.
5. If the extract is mixed with human blood serum, its avticity is rapidly lost (fig. 3: 4) but not if the blood serum had previously been treated with eserine sulphate 1 : 100,000 (fig. 3: 5).
   (To 3 ml extract of pH 6.9 0.5 ml blood serum was added. After 5 minutes the protein was precipitated with trichloracetic acid and filtered away. The excess of trichloracetic acid was removed through repeated washings with ether. The extract was freed from ether through evaporation, neutralized and tested.)
6. The titration of the extract on *m. rectus* of frog, on eserinized leech muscle and on frog's heart gave the following values respectively: 0.090, 0.084, and 0.096 γ per gram of tissue. The proportion between muscarinelike and nicotinelike activity was thus found to be the same for acetylcholine as for the active substance to be identified.

As pointed out by GADDUM (1936, pag. 68) acetylcholine is the only known substance that complies with these tests.

Source of the Acetylcholine Liberated by Heat and Acids.

In the alcoholic extracts from resting organs little or no free acetylcholine is found, but at treatment with heat and acids an active substance is formed which has been identified as acetylcholine. As to the source of this acetylcholine the following possibilities can be discussed:

1. the liberated acetylcholine is preformed in the organs but is held within undamaged cell membranes. Therefore no pharmacological activity is developed when the extract is tested. Acids and heat desintegrate the cell membranes and the preformed acetylcholine is liberated. This theory has been forwarded by TRETHEWIE (1938).
2. The tissue acetylcholine may be present as an adsorbate to proteins, and is liberated when the protein is denaturated by heat and acids, (*vide* QUASTEL and co-workers, 1939). KAPFHAMMER and co-workers (1931) also came to the opinion that part of the tissue acetylcholine is adsorbed to protein. HEIM (1939) found that addition of protein to the perfusion fluid lessened the sensitiveness of isolated hearts to acetylcholine. He concluded that protein adsorbed acetylcholine.

3. the acetylcholine formed is synthetized from choline and acetic acid.
4. the acetylcholine is liberated from a labile complex.

In the earlier experiments on "precursor" the formation of ach has only been shown in cell-holding extracts or in whole organs (vide pag 77). In the experiments reported above, we have shown this formation of acetylcholine in an extract that was free from cells. Alternative 1 is thus contradicted and thereby also the objections, which TRETHEWIE has raised against the experiments of QUASTEL et al.

Alternative 2 is contradicted by the experiments 5 and 6. As shown by experiment 5, the sulphosalicylic acid test for proteins was negative in an extract, which contained no free acetylcholine but formed 4.6 γ acetylcholine chloride per ml after being acidified and heated. The sulphosalicylic acid test is positive when the protein concentration amounts to more than 1 : 200,000. The extract used in experiment 5 must have contained less than 5 γ protein per ml. If the molecular weight of protein is calculated as 30,000, this means that the extract has contained less than one molecule of protein per 100 molecules of "bound" acetylcholine.

The alternative 2 is also contradicted by the fact, that liberation of acetylcholine also may be demonstrated in extracts deproteinized with trichloracetic acid. If a trichloracetic acid extract is prepared in full accordance with the description of CHANG and GADDUM (1933), almost all "tissue acetylcholine" is found as free, active acetylcholine. If, however, the extract is prepared with the same precautions — as far as possible — which we have used for the extraction with alcohol, and if the excess of trichloracetic acid is removed as rapidly as possible, the extract contains almost no active acetylcholine; after acidifying and heating about the same amount of acetylcholine appears as earlier found in the alcohol extracts (*vide* experiment 6).

Alternatives 3 and 4 remain for discussion. Is the formation of active acetylcholine in our extracts due to a synthesis or to a breakdown of a labile complex? An enzymic synthesis does not seem to be very probable. The extracts are, as shown by experiments 5 and 6, free from cells and protein. Also the non-enzymic formation of acetylcholine from choline and acetic acid seems to be impossible. When choline is boiled with acetic acid, no acetylcholine is formed. This view is also supported by the fact that the

acetylcholine forming fraction of our inactive extracts may be separated from choline, acetid acid and preformed acetylcholine. These last mentioned substances are readily soluble in acetone and cannot be precipitated with barium salts. When, however, the inactive extract is treated with acetone or with soluble barium salts a precipitate is formed, which contains no acetylcholine but liberates acetylcholine when treated with heat and acids (experiment 7).

The liberation of acetylcholine is therefore most probably due to the breakdown of a complex. As we know nothing about its chemical constitution we call it acetylcholine precursor.

Experiment 5.

16 rabbits' hearts, weighing 96 grammes, were extracted with 250 ml 96 per cent alcohol as described above. The extract was evaporated in vacuo to about 1 ml; redissolved in alcohol, centrifugated and again evaporated in vacuo to 0.5 ml. The remainder was dissolved in 5 ml Ringer solution and insoluble matters were removed by centrifugation. On part of the solution the sulphosalicylic acid test was made with negative results. After neutralization, part of the solution was tested for free acetylcholine on eserinized *m. rectus abdominis* of frog. Another part was acidified to pH 5.5 and heated to 80° for 45 minutes. After neutralization it was tested for acetylcholine.

free acetylcholine $= 0.14\ \gamma$ per ml $= 0.007$ per gram of tissue.
precursor acetylcholine $= 4.6\ \gamma$ per ml $= 0.24\ \gamma$ per gram of tissue.

Experiment 6.

4 rabbits' hearts, weighing 23.5 grammes and frozen with solid carbon dioxide and ether immediately after the animals had been shot, were pressed to thin disks and placed in a cooled mortar containing 50 ml 20 p.c. trichloracetic acid, which was partly frozen. The disks were cut into fine pieces and ground with quartz sand. Insoluble matters were removed by filtration and washed twice with 4 p.c. trichloracetic acid. The filtrate was made just neutral to kongo paper and then washed with large amounts of ether until it became neutral to litmus. 5 drops of 2-N acetic acid was added and the solution was evaporated to about 2 ml at 30° C and at reduced pressure. Ringer solution was added to make 10 ml. The extract was tested for free acetylcholine on *m. rectus* of frog. Then it was acidified to pH 5.5 and heated to 80° C for 45 minutes and tested for acetylcholine.

free acetylcholine $= 0.028\ \gamma$ achCl per gram of tissue.
precursor acetylcholine $= 0.17\ \gamma$ » » » » »

Some Observations on the Chemical Properties of the Precursor.

Solubility: The method for the determination of the precursor, described on page 80, includes extraction with 96 p.c. alcohol (i.e. in reality alcohol diluted with water from the organs), re-dissolving in absolute alcohol, washings with ether and finally dissolving in water or Ringer solution. We have controlled that no precursor is lost at the re-dissolving in 99.6 per cent alcohol or at the washings with ether. We may therefore conclude that the precursor — like free acetylcholine and free choline — is soluble in water, absolute alcohol and mixtures of water and alcohol, but insoluble in ether. We also found the precursor to be insoluble in petrol ether. Contrary to free acetylcholine and free choline the precursor is insoluble in acetone; a water solution of the precursor may be precipitated with acetone, (*vide* experiment 7). Acetone is thus a medium which can be used to separate the precursor from acetylcholine and choline. We also found that the precursor can be precipitated from a watery or alcoholic solution with soluble barium salts. Precipitation occurs at pH 4.5 as well as at pH 7.

Experiment 7.

5 rabbits' hearts were extracted with 96 p.c. alcohol according to the method described above. This primary extract was divided into 2 equal portions. The first portion was treated in full accordance with the description on page 81 and its contents of free acetylcholine and precursor were titrated on eserinized *m. rectus* of frog. To portion II was added free acetylcholine in an amount corresponding to 0.26 γ acetylcholine per gram of tissue. The extract was evaporated as usual and re-dissolved in absolute alcohol. This was transferred to a centrifuge tube with a capacity of about 100 ml. Water was added and the solution was evaporated to 1—2 ml at 30° C. and at reduced pressure. Then 80 ml waterfree acetone were added. A white precipitate appeared, which was centrifugated 15 minutes at 3500 r./min. The acetone was poured into an evaporation vessel; 5 ml water and 2 drops of 2-N acetic acid were added and the acetone was evaporated at 30° C and at reduced pressure until only the added water was left. The watery solution was centrifugated, neutralized and tested for free acetylcholine and precursor.

The acetone precipitate was dissolved in 5 ml Ringer solution and its contents of free acetylcholine and precursor were determined on

Portion I

free acetylcholine = 0 (= less than 0.01 γ per gram of tissue)
precursor = 0.19 γ per gram of tissue

Portion II

a) acetone soluble
free acetylcholine = 0.21 γ per gram of tissue = added amount
precursor = 0

b) acetone precipitate
free acetylcholine = 0
precursor = 0.17 γ per gram of tissue.

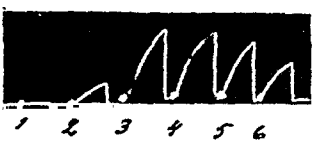

Fig. 4. (Stability of the precursor).

Fig. 4 shows the formation of acetylcholine from the precursor and the following hydrolysis of the formed acetylcholine, which results when a precursor holding extract is heated after addition of HCl.

The extract was prepared as described for the "A-portion" in experiment 2. It was then acidified to pH 5.5 under the control of a glass electrode apparatus and put on a boiling water bath. After varying periods (0, 5, 10, 15, 30 and 45 minutes) corresponding to points 1, 2, 3, 4, 5, and 6 respectively, samples were drawn, cooled, neutralized and their contents of free acetylcholine were tested on an eserinized m. rectus abdominis of frog. In the experiment represented above the same amount of extract was added. Between every addition the kymographion was arrested for 10 minutes.

Stability.

As we have learnt from the experiments above, the precursor is a very labile substance. The breakdown of the precursor is a more rapid process than even the hydrolysis of the liberated acetylcholine in choline and acetic acid — otherwise the amount of free acetylcholine would not have increased. On the other hand the lability of free acetylcholine is so great that a loss through hydrolysis is inevitable when the extract is acidified and heated in order to liberate acetylcholine from the precursor. This is illustrated by figures 4 and 5. In the experiment represented by fig. 4 a precursor-holding extract was acidified and heated, and the concentration of free acetylcholine determined at varying intervals. As seen from the fig., the first samples show a rapid increase of free acetyl-

choline. In this stage the formation of acetylcholine from the precursor is greater than the loss through hydrolysis. Later the apparent acetylcholine formation comes to a standstill, and if the solution is heated any longer, the acetylcholine concentration decreases. The same experimental findings are seen in fig. 5. It is quite evident that the maximal value of free acetylcholine found at these experiments is lower than the amount of "bound"

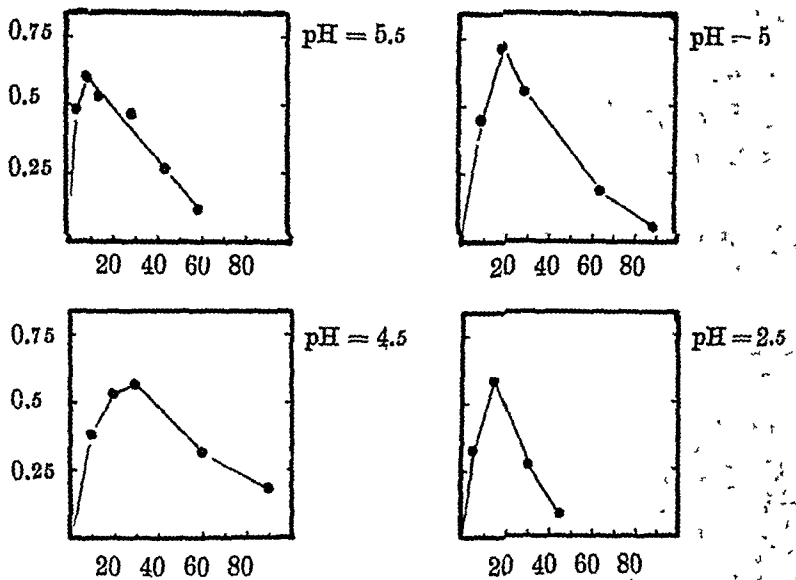

Fig. 5.

Fig. 5 shows the formation of acetylcholine from the precursor and the following hydrolysis of the acetylcholine formed, when a precursor holding extract is heated at varying acidity.

y = amount of acetylcholine found expressed as γ of acetylcholine chloride per ml of extract. x = period of hydrolysis in minutes.

The extract was prepared from rabbits' hearts a[illegible] ance with the "A-portion" in experiment 2. Free a[illegible] was removed through washings with acetone. The e[illegible] solution and divided into equal parts. The acidity [illegible] with HCl under the control of a glass electrod[illegible] pectively. They were heated on boiling water bath. [illegible] were drawn, immediately cooled, neutralized and their contents of free acetylcholine were titrated on eserinized m. rectus abdominis of frog.

acetylcholine originally present in the extracts as precursor. During the period of time which is necessary to reach the maximum amount of free acetylcholine, part of the acetylcholine is hydrolysed. In the experiments for this communication we have determined the precursor as the amount of free acetylcholine liberated at treatment with acids and heat. Therefore, all quantitative data on the precursor given in this paper are too low.

Not only are our values on precursor too low, but all quantitative data in the literature on "tissue acetylcholine" in resting organs are too small. As will be more closely discussed below, all tissue acetylcholine in resting organs is present as precursor. The acetylcholine found derives from the precursor, which is broken down during the analysis under the influence of various agents, e.g. trichloracetic acid. Nobody knows if this breakdown reaches its maximum point or goes any further.

In order to evade losses of the formed acetylcholine we studied the breakdown of the precursor at varying acidity. In this regard our experiments were not successful. At pH 4, at which point free acetylcholine is most stable (*vide* fig. 1), the precursor also seems to be most stable. The liberation of free acetylcholine from the precursor seems to be a process which — like the hydrolysis of free acetylcholine — is catalyzed by OH as well as H ions. If the pH of the extracts is moved to the acid or the alkaline side of pH 4, the precursor is rapidly broken down, (*vide* fig. 5). As seen from fig. 5, the breakdown of the precursor seems to be 5—6 times more rapid than the hydrolysis of the acetylcholine formed. It was, however, not possible to make a correction for the inevitable loss of free acetylcholine. The shape of the curve was found to be highly influenced not only by the acidity but also by the unknown concentration of precursor and the initial amount of free acetylcholine eventually present. To these difficulties came that it was impossible to use buffer solutions at the acidifying of the extracts as the buffer substances disturbed the biological assay of acetylcholine.

Is any Free Acetylcholine Present in Resting Tissues?

The presence of free acetylcholine in extracts from a great variety of organs has been shown by many authors. Chemical as well as pharmacological methods have been used for the identification. It has hitherto not been published any experimental investigation on the question if the free acetylcholine, found in the extracts, exists preformed in the tissues, although pharmacologists have been of opinion that tissue acetylcholine in some unknown way must be inactive (Chang and Gaddum, 1933; Beznák, 1933). On the basis of their experimental findings, some authors have assumed the presence of a complex, assumed to play a rôle at the synthesis of acetylcholine from choline and acetic acid. But also

these investigators found the usual amounts of free acetylcholine beside the formation of extra acetylcholine due to breakdown of the assumed complex or to a synthesis. The starting point for our experiments was somewhat different. The main question was: does there exist any preformed, free acetylcholine at all in the tissue; if not, in what state does tissue acetylcholin exist? The question if there exists free acetylcholine or not in resting tissues has more than academical interest. As long as we accept the presence of free acetylcholine in resting tissues and as long as we determine the tissue acetylcholine as free acetylcholine, we have no possibilities to study within the organs the very process of acetylcholine liberation at stimulation.

In the previous chapters we have shown the presence of an inactive substance, an acetylcholine precursor, which liberates active acetylcholine in the test tubes. No — or almost no — free acetylcholine was found, but the amounts of the precursor was of the same magnitude as the amounts of free acetylcholine found with earlier methods. This supports the assumption, proposed in the introduction of this paper that the free acetylcholine found in the extracts of earlier methods, in reality derives from the complex, which breaks down during the analysis. To secure this opinion, it is necessary to show:

1) that practically no free acetylcholine is lost at our method,
2) that the conditions of earlier methods induce a breakdown of the precursor.

It does not seem very probable that free acetylcholine should be lost at our method. In most cases we have found no preformed acetylcholine in extracts which contained large amounts of precursor. As far as our knowledge goes, the breakdown of free acetylcholine is favoured by the same conditions as the liberation of free acetylcholine from the precursor, but the latter process is more rapid. An improper treatment inducing a loss of free acetylcholine would therefore in the first place induce an even greater formation of free acetylcholine from the precursor. As seen in experiment 6, an added amount of free acetylcholine is quantitatively refound, while the extract itself contained no preformed acetylcholine.

According to the view developed in this paper, earlier methods must induce a breakdown of the precursor. The most commonly used extraction fluid is 10 p.c. trichloracetic acid. This acid is very strong, comparable with sulphuric acid; its dissociation constant

is given as $2 \cdot 10^{-1}$. An extract made from 1 vol. muscle pulp and 2 vol. 10 p.c. trichloracetic acid has an acidity that is stronger than pH 1. Experiments with known amounts of precursor, dissolved in trichloracetic acid, showed that at pH 1 and at room temperature about 80 per cent was broken down in 60 minutes. The same is seen from experiment 8. We may therefore conclude that not only the discussion of pharmacological items but also direct, biochemical evidences favour the opinion that resting organs contain no free, preformed acetylcholine but a labile compound, which is the source of the acetylcholine found with earlier methods.

Experiment 8.

5 rabbits' hearts, weighing together 28 grammes and frozen in solid carbon dioxide and ether, were pressed to thin disks and laid in a cooled mortar containing 60 ml 10 p.c. trichloracetic acid, partly frozen. The disks were cut into fine pieces and ground with quartz sand. The extract was cleared through centrifugation and then divided into 2 equal portions. The first portion was immediately made neutral to kongo red and then washed with ether until it became neutral to litmus. Its volume was evaporated to 10 ml at 30° C and at reduced pressure. Free and "bound" acetylcholine was determined. The temperature of the other portion was allowed to rise to 19° C and then it was treated in full accordance with the description of CHANG and GADDUM (1933). Free acetylcholine and precursor was titrated on eserinized *m. rectus* of frog.

Portion 1:

free acetylcholine = 0.03 per gram of tissue
precursor = 0.18 per gram of tissue.

Portion 2:

free acetylcholine = 0.14 γ per gram of tissue
precursor = 0.05 γ per gram of tissue.

Summary.

The presence of free acetylcholine in extracts from various organs has been secured by many investigators using chemical as well as biological methods for the identification. The discussion of pharmacological evidences allowed the conclusion, however, that this free acetylcholine found in the extracts is not preformed in resting organs. The discussion also supported the working hypo-

thesis that the free acetylcholine in the extracts is formed throug breakdown of a preformed complex. It was assumed that th breakdown of this complex under liberation of free acetylcholin is favoured by the methods hitherto used for the determinatio of acetylcholine in tissues.

The experiments presented in this communication have give the following results:

1) Resting organs were frozen with liquid air and extracted at low temperature with ethyl alcohol. The alcohol was evaporate and the extract concentrated in vacuo. This extract was free fro cells and from proteins. It contained no free acetylcholine, althoug added acetylcholine was quantitatively refound. If the extrac was acidified and heated, an active substance was formed, whic was found to be acetylcholine.

The same results were obtained with trichloracetic acid extract if only the organs to be extracted were frozen with liquid air, th extraction made at a low temperature, and if the excess of trichlor acetic acid was removed as soon as possible.

2) The *formation of acetylcholine at the heating of acidifie* extracts is not due to a synthesis. The extracts are free from cell and as far as known free from proteins; addition of choline an acetic acid has no influence on the formation of acetylcholine; th formation can also be shown in extracts where all free choline ha been removed through extraction with acetone. The formation o acetylcholine is due to breakdown of a labile complex compound which we call "precursor" as we know nothing about its chemica constitution.

3) The precursor was found to have the following properties Like free acetylcholine it is soluble in water, in absolute alcoho and in mixtures of water and alcohol, and insoluble in ether an petrol ether. Unlike free acetylcholine and choline it is insoluble in acetone and can be precipitated with acetone from a watery solution.

It is precipitated with barium acetate from neutral or slightly acid watery or alcoholic solutions.

It is relatively stable at pH 4. At other acidities it breaks down liberating free acetylcholine. This process is considerably more rapid than the hydrolysis of free acetylcholine.

4) The free acetylcholine earlier found in extracts from resting organs is formed through breakdown of the precursor during the analysis.

The experiments allow the following conclusion: Resting organs contain no preformed acetylcholine. Tissue acetylcholine is present as an integral part of a labile complex compound, which differs from free acetylcholine through its chemical properties.

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Citric Acid Content in Meal of Certain Cereals and Leguminous Plants.

A Contribution to the Question of Exogenous Citric Acid.

By

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While citric acid has long been considered a typical vegetable acid, research in the last decade has shown that it also plays an important part in animal organism. In the form of citrates it is found in varying amounts in practically all fluids and tissues of the mammalian organism.

The question arises as to where the citric acid supply in the mammalian organism originates. It has already been shown that it is partly supplied to the organism through food, "exogenous citric acid", partly formed in the organism, "endogenous citric acid". In the exogenous citric acid we must include that formed in the digestive tract by fermentation processes, which is later reabsorbed.

The food of even a new-born mammal contains citric acid. As shown by HENKEL (1886) there is a considerable amount in milk. On being weaned, the young herbivorous mammal finds citric acid in vegetable food. Calves, for example, get the same amount of citric acid from green grass and clover as they formerly obtained from cows milk (WESTERLUND). Actually the animal on vegetarian diet obtains citric acid from all kinds of food. What differentiates typical citric acid fruits from other vegetables is thus partly the much higher amounts in which citric acid is found in the former, partly its presence as free acids.

Citric acid is also of biological interest as a constituent of secretion and excretion of animal organisms, something already apparent from its presence in milk. That it also is a normal constituent of urine, however, was first shown by AMBERG and MC CLURE (1916) and later by ÖSTBERG.

Since then the appearance of citric acid in different secretions has been the object of exhaustive investigations. With special reference to the quantitative aspects of the question, however, it remains to analyze the essential deposits where citric acid appears, as income and output in metabolism, and so obtain a balance sheet for the substance. As a contribution to the knowledge of the amount taken in in this calculation, the results of a series of analysis are given here, concerning the citric acid content of certain important cereals and leguminous plants, or rather, determined more exactly, from the finely sifted meal from these as it is used in our food.

In carrying out these experiments the convenient and sensitive method for citric acid determination worked out by PUCHER, SHERMAN and VICKERY ("PSV-method") is used. The enzymatic method ("T. method", see MÅRTENSSON) of THUNBERG is not suitable for determining citric acid in solid substances.

No account of the PSV-method will be given here as it is described in detail in easily accessible Scandinavian scientific literature (HALLMANN and KRUSIUS). Here it is only mentioned that the decolourization of the potassium permanganate took place while using hydrogen peroxide solution and taking into consideration the precautions specially emphasised by DICKENS to avoid any surplus of hydrogen peroxide. Furthermore, the seedpowder was allowed to stand unstirred in water for 12 hours before the addition of trichloracetic acid. Control experiments show that the amount of pre-formed citric acid is nevertheless unchanged, and also, that the precipitation of proteins is not affected when a smaller amount than usual of trichloracetic acid is used.

The experimental material consisted of wheat, rye, oats, barley and maize, as well as the seeds of 11 different kinds of legumes. The seed material is ground by a roller mill with grooved rollers of stainless steel, the distance between which can be varied. If necessary the material is passed through the mill repeatedly. The powder obtained is then sifted through a no. 20 sieve, i.e. a sieve with 20 threads per cm. On the basis of preliminary experiments portions of the sifted powder (flour) are then weighed and the amount of their citric acid content found to be between 0.1 and 1.0 mg. Before final analysis the powder remained undisturbed for 12 hours in 50 ml distilled water with a temperature of + 4°C.

The citric acid values obtained are given below (table 1).

Thus, while all the seeds examined contain citric acid, it is present in very different amounts in the different kinds. The lowest

Table 1.

Citric acid content in per cent.

A. *Cereals.*		B. *Seeds of leguminous plants.*	
Avena sativa	0.01	Lathyrus sativus	0.47
Hordeum sativum	0.03	Lupinus angustifolius	0.97
Secale cereale	0.03	Lupinus luteus	1.80
Triticum sativum	0.01	Medicago sativa	0.60
Zea mays	0.20	Phaseolus multiflorus	2.14
		Pisum sativum	0.79
		Tetragonolobus purpureus	1.18
		Vicia faba	0.33
		Vicia sativa	0.39

content, 0.01—0.03 %, is found in flour from ordinary cereals. The corresponding value for Zea mays is 0.2 per cent. Citric acid content in seed powder from legumes as a rule is higher than in that from cereals. The variations here are considerable. Seed powder from Vicia faba thus contains 0.33 per cent while peas contain more than double the amount, and Phaseolus multiflorus more than 2 per cent.

In the following an account is given of a number of experiments on the determination of possible variations in the citric acid content of germinating seeds. The seeds were grown in the dark to prevent new formation of organic substance through assimilation.

Materials used were: Original Petkus Rye (Weibull), Eroica Wheat (Weibull), Original Balder Barley (Weibull), Original Bambu Oats (Weibull), Sugar Maize (Norman's Seed Co.).

Ten portions (each 0.5 gram) of each kind of seed are weighed. Each portion is evenly distributed in a separate Petri dish, the base of which is covered with damp filter paper. The dishes are then placed in the dark at room temperature. After two days the citric acid content is determined in portion 1; some days later portion 2 is analyzed and so on.

For analysis the germinating seeds are crushed and stirred in distilled water. The stirred mass stands overnight and then is treated with trichloracetic acid for protein precipitation. The PSV-method is used for analysis. The contents of the remaining nine dishes are treated in the same way besides which a control determination is carried out with non-germinating seeds.

While experimenting with rye and wheat a complication arose with the development of a kind of mould forming citric acid, judging by the increase in citric acid in the cases where mould developed. The complication was prevented by treating the cereals with calomel which does not seem to affect the germinating ability of the seeds. The results obtained are summarised in the diagram below, where the abscissa denotes germination time per 24 hours and the ordinate, citric acid content in μg per gram.

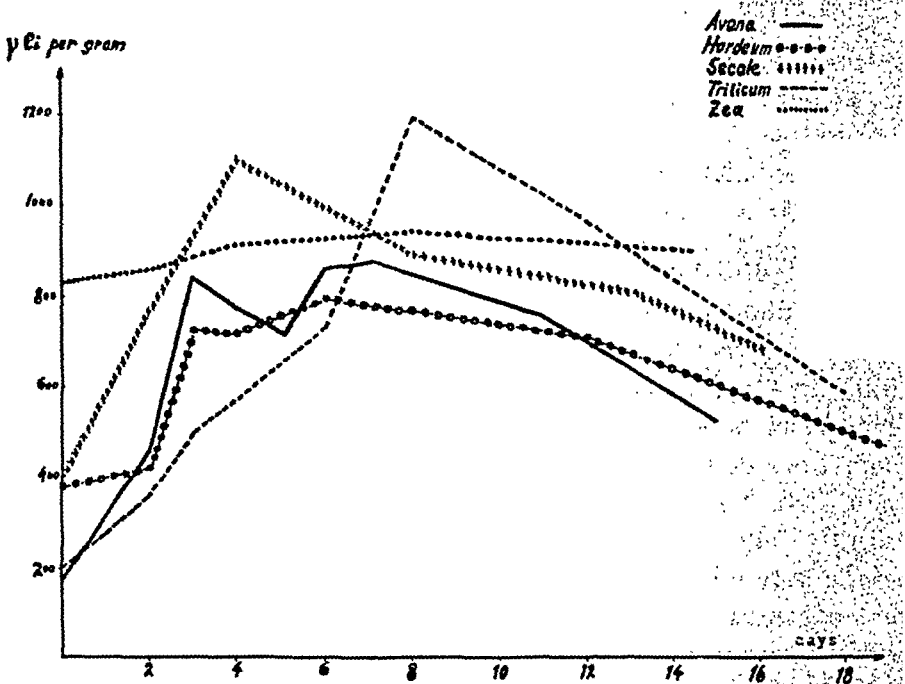

Fig. 1.

All curves show that during germination period the citric acid content rises initially and then decreases. The highest values observed during germination expressed in per cent of the initial values are: for wheat 600 per cent

rye	275 per cent
barley	208 per cent
oats	464 per cent
maize	115 per cent.

The citric acid content during germination rises most considerably in wheat and least in maize. The low value for maize is possibly due to the fact that the citric acid content for this cereal is fairly high from the beginning. As germinating plants under the

present experimental conditions have no possibility of increasing their supply of organic substance, the contribution to citric acid obtained must be of endogenous origin.

The distribution of the total amount of citric acid present in germinating plants in their different parts and at different stages of their development has been investigated in barley and oats. At different times after the cereals have been set to germinate they are cut into two parts, one containing the shoot, the other the rest, i.e. the root and the seed freed from the shoot. In barley the shoot was found to contain a proportion of the total citric acid content increasing with time. This proportion on the 6th, 12th and 19th day after the start of germination was 15, 45 and 80 per cent respectively. After removal of the shoot the proportion in the rest of the plant decreased, being at the above times 85, 55 and 20 per cent respectively. A similar determination for oats revealed that the percentage of the total amount of citric acid contained in the shoot determined on the 4th, 7th, 15th and 18th day increased likewise, being 25, 33, 51 and 70 per cent respectively. On the other hand the values for the rest of the plant decreased to 75, 67, 49 and 30 per cent of the total citric acid content.

Summary.

As a link in the systematic examination of citric acid balance in the animal organism, the citric acid content is determined in flour from wheat, rye, barley, oats and maize and from various leguminous plants. Citric acid was found in all the flour and seed powder examined, less in the cereals, more in the leguminous plants.

Cereals grown in the dark showed a considerable increase in citric acid after some days which, however, decreased with continued growth of the plant. During germination the relatively highest amount of citric acid was found in the shoot.

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A Method for Quantitative Determination of Acetylcholine Precursor and Free Acetylcholine in Tissues.

By

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Received 24 April 1944.

In previous communications (ABDON and HAMMARSKJÖLD, 1939, 1944) we have shown that all tissue acetylcholine in resting organs is present as an integral part of a labile complex compound, which differs from free acetylcholine through certain, well defined chemical properties. The complex is labile and when exposed to acids and heat it breaks down liberating free acetylcholine. We call the substance "acetylcholine precursor" or, shortly, "precursor". The free acetylcholine found in resting tissues with earlier methods in reality derives from the precursor, which is broken down during the analysis under the influence of the very acid extraction fluids generally used. We assume that the precursor is the inactive and labile compound from which free acetylcholine is released in the organs during various physiological conditions. To be able to study this physiological acetylcholine liberation it was, however, necessary to construct a more reliable, quantitative method for the determination of the precursor as well as the liberated acetylcholine in tissues.

In our earlier experiments we determined the precursor as the amount of free acetylcholine liberated under the influence of heat and acids under certain fixed conditions. As emphazised in the earlier communications, all values obtained with this method are too low and the method should not be used for strictly quantitative studies. The precursor is most stable at about pH 4, just

as free acetylcholine. If free acetylcholine is to be liberated from the precursor, this has to be done at a more acid or more alkaline reaction. It is inevitable that part of the liberated acetylcholine is further hydrolyzed into acetic acid and choline and thus lost at the biological titration. When a precursor-holding solution is acidified and heated, there is first a rapid rise in the acetylcholine concentration. This soon reaches its maximum and if the solution is heated any longer, the concentration of acetylcholine falls to zero. It is evident that the maximal concentration of acetylcholine found must be smaller than the amount originally present as precursor (see ABDON and HAMMARSKJÖLD, 1944). We tried to make a correction for the inevitable losses, but too many factors were found to influence, *e. g.* the initial concentration of precursor and free acetylcholine. It ought to be emphazised that all earlier methods for the determination of "tissue acetylcholine" are impaired with the same inaccuracy, as also in these cases the acetylcholine determined is formed through breakdown of the precursor.

In constructing a new method for the determination of the precursor we made use of our finding that the precursor is insoluble in acetone contrary to free acetylcholine and choline. The tissue extract is now divided into two portions: the acetone soluble fraction, containing free acetylcholine and choline, and the acetone precipitate which contains the precursor. The precursor is then broken down — not to acetylcholine which cannot be done without losses of unknown magnitude — but to choline, and then this choline is reacetylated to acetylcholine, an operation which can be made with great accuracy. Below this method is described, first as a summary and then with necessary details and experiments.

Method.

I. Extraction.

a. At the killing of the animal etc. the organs are frozen with liquid air or a mixture of ethyl ether and solid carbon dioxide. The organs are kept in a Dewar flask with solid carbon dioxide until worked up.

b. The frozen organs are pressed into thin disks and laid in a mortar with twice their weight of 96 p. c. ethyl alcohol, which

has been previously cooled to about — 20°. The disks are cut into fine pieces and ground with quartz sand. The operations are made in cold. The extract is filtered through sintered glass filters and centrifugated.

c. The clear extract is acidified with 0.5 ml concentrated acetic acid per 100 ml extract. Its volume is reduced to 1—2 ml at 30° and at reduced pressure. The residue is washed twice with large amounts of ether, which is drawn off with a suction pump after sedimentation. Remaining ether is evaporated in vacuo.

d. The extract is then diluted with at least its ten-fold amount of absolute ethyl alcohol. Insoluble matters are removed by centrifugation. 4 ml of distilled water and a drop of concentrated acetic acid are added and the volume is reduced at 30° and at reduced pressure to a volume of about 1 ml.

II. Separation of the Precursor from Free Acetylcholine and Choline.

a. The product from I d is precipitated with large amounts (about 80 ml) of water free acetone and centrifugated. The supernatant clear acetone is decanted and used for the determination of free acetylcholine and choline (II b). The precipitate is dissolved in 0.5 to 1 ml of distilled water and again precipitated with 80 ml acetone, centrifugated and the acetone is decanted. This is repeated for a third time.

The precipitate, which contains the precursor, is dissolved in 0.5 ml distilled water, a drop of 2.5 p. c. NaOH is added and the solution is heated on waterbath for 2 minutes. The liberated choline is then re-acetylated to acetylcholine (III a).

b. To the first acetone portion from II a. is added 5—10 ml of distilled water and a drop of acetic acid. The acetone is removed at 30° and at reduced pressure. The remaining watery solution is neutralized and its contents of free acetylcholine are titrated. The watery solution is also used for determination of choline.

c. If choline is to be determined, the watery solution from II b is washed 3 times with petrol ether (Bp 33°—45°), which is drawn off with a suction pump. Petrol ether remaining in watery solution is removed in vacuo. A measured part is acetylated (III a).

III. Acetylation.

a. To the precipitate which has been treated with NaOH (from II a) is added 10 ml of acetic anhydride (pro analysi, free from homologes).

To a measured part of the watery solution from II c, not exceeding 1 ml in volume, is added the 20-fold amount of acetic anhydride.

The solutions are transferred into long test tubes with a capacity of 40—50 ml. These are covered with tinfoil and heated on boiling water bath for 10 minutes.

b. The greatest part of the excess of acetic anhydride is removed by evaporation at 60—80° and at reduced pressure. When 1—2 ml are left, 10 ml of distilled water are added and mixed with the anhydride. The remaining acetic anhydride is removed through repeated washings with ethyl ether, (10 times), which is drawn off with a suction pump after sedimentation. Ether in watery solution is removed in vacuo. The reaction should now be neutral to litmus. The volume is made up to 10 ml. The contents of acetycholine are measured on eserinized *m. rectus abdominis* of frog.

I. Extraction.

When the organs are taken for examination they are as soon as possible frozen with liquid air or a mixture of ethyl ether and solid carbon dioxide. The contents of precursor in some organs, *e. g.* hearts of rabbit or dog rapidly decrease if they are not frozen as rapidly as possible. Striped muscles of frog, on the other hand, can be allowed to lie at room temperature in oxygenated Ringer solution or in a moist chamber for 2 hours without losing any precursor, provided that they are not mechanically injured. Injury causes breakdown of the precursor. It is, therefore, necessary that the organs are kept frozen when they are worked up and when they are extracted, until all proteins are precipitated by the extraction fluid.

In our previous communication we have shown that the precursor as well as free acetylcholine and choline is readily soluble in alcohol water, mixtures of alcohol and water, and in trichloracetic acid, but insoluble in ether and in petrol ether. As a matter

of principle it is possible to use trichloracetic acid for extraction; properly handled it may give the same yield of precursor and free acetylcholine as ethyl alcohol. Extraction with alcohol has many advantages. It makes it possible to work at such low temperatures as —20°, at which temperature any enzyme which may split or synthezise the precursor or acetylcholine most probably is completely inhibited. After addition of acetic acid the alcoholic extract has a slightly acid reaction, at which the precursor as well as the free acetylcholine were found to be rather stable. Trichloracetic acid gives an extract of very acid reaction, about pH 1, which accelerates the breakdown of the precursor. The alcohol extract has further the advantage that it can be concentrated more rapidly and under more favourable conditions with regard to the stability of the labile choline compounds.

The still frozen organs are pressed into thin disks with a Buchner press. The disks are laid in an agat or iron mortar, which has previously been cooled with solid carbon dioxide. The disks are pounded into small pieces with a cooled pestle. Then cooled 96 p. c. ethyl alcohol is added; 2 ml for every gram of tissue. The temperature is allowed to rise to about —20°. At this low temperature the pieces of organ soften so that it is possible to cut them into finer pieces with a pair of scissors, if necessary. A little quartz sand is added and the organs are thoroughly ground. The temperature is then allowed to rise to about zero and the contents of the mortar are stirred for about 5 minutes. The extract is filtered through sintered glass filters (Jena No. G1 or G2) with the aid of a suction pump. The insoluble remnant is thoroughly washed with 1 ml of 96 p. c. alcohol for every gram of tissue. The filtrate is centrifugated 15 minutes at 3,500 r/min.

The extract has a slightly acid reaction, chiefly caused by carbonic acid, which evaporates at the further treatment of the extract. As mentioned above, the precursor as well as the free acetylcholine is most stable at about pH 4. In the preliminary experiments this acidity was roughly achieved through addition of HCl. Later we found that addition of acetic acid does not bring about acetylation of choline (see experiment 6) or any synthesis of precursor. Now we therefore adjust the reaction by addition of 0.5 ml of concentrated acetic acid for every 100 ml of extract.

The initial volume of this primary extract should not exceed 120—140 ml, corresponding to about 35 gram of tissue. If the

amount of tissue is larger, it ought to be divided and each portion treated separately until the precipitation with acetone.

The volume of the primary extract is then reduced to 1—2 ml by means of evaporation at reduced pressure and at 30°. To this end the extract is quantitatively transferred into an evaporation flask made of thick glass and with a capacity of 250—300 ml. To avoid losses due to foaming the flask is provided with a long neck. At the upper end of the neck a side tube is inserted which is connected with a water suction pump. The mouth of the neck is tightened with a rubber stopper through which a glass tube is inserted. This is drawn out into a fine capillary which reaches the bottom of the flask. The flask is placed into a water bath with a temperature of 30°. A brisk aeration is set up through the capillary and extract is evaporated to 1—2 ml. If foaming should become disturbing, the extract is shaken with ether in the flask. After sedimentation the ether is drawn off with a suction pump, a drop of concentrated acid is added and the evaporation is continued.

When the volume has been reduced to 1—2 ml, the extract is washed twice with about 100 ml of ether, which is drawn off after sedimentation. The ether removes neutral fats and also the main part of some choline compound of phosphatide nature, which would otherwise disturb the determination of choline. The precursor, acetylcholine, and choline are quite insoluble in ether; we have found no losses after washings with ether, and the ether contained no precursor and no free acetylcholine. The ether also removes some water and the extract thus becomes nearly dry.

The crude extract, which is obtained in this way, also contains impurities which may disturb the biological titration of acetylcholine, *e. g.* potassium salts (see GADDUM, 1936) and adenosine compounds (ABDON, 1942). These impurities are practically insoluble in 90—100 p. c. alcohol. It is sometimes stated in the literature that the primary extraction with alcohol has the advantage of giving an extract which is free from potassium salt. The water from the organs is, however, enough to dilute the extract to such an extent that practically all potassium of the organ is extracted. The concentrated extract is dissolved in 10—12 ml absolute alcohol. Insoluble matters are removed by centrifugation. The alcohol is transferred into an evaporation flask. Distilled water is added to make the alcohol about 60—70 p. c.; a drop of concentrated acetic acid is added and the volume is reduced

to 1—2 ml at reduced pressure and at 30° C. If water is not added before evaporation, a great part of the precursor and the acetylcholine will be lost (experiment 5).

II a. Separation of the Precursor from Acetycholine and Disturbing Choline Compounds.

At the method for determining the precursor proposed in this paper the precursor is broken down to choline and then re-acetylated to acetylcholine, which is biologically determined. It is, therefore, necessary to remove not only free acetylcholine and choline but every choline compound that may form acetylcholine at the process of acetylation. The main representative of this group of choline compounds is lecithine, but there are probably other members of this group. Although part of the disturbing choline compounds have been removed at the washings with ether, the extract yet contains acetylcholine, choline and other compounds beside the precursor. The separating is made by means of acetone, which precipitates the precursor, but leaves the other compounds in solution. Lecithine, which is insoluble in acetone when pure, is soluble in the presence of impurities as fatty acids (MacLean, 1918). In the preliminary experiments we precipitated the acetone soluble fraction with barium salts in order to purify the precursor. This was found to be unnecessary. The separation is performed in the following way: 3—5 ml of waterfree acetone are added to the watery extract, which is obtained after the second alcohol extract has been evaporated. The mixture, which is as a rule still quite clear, is transferred to a centrifuge tube with a capacity of about 100 ml. The evaporation flask is rinsed with a little acetone three times. The volume of acetone is made up to about 80 ml. A white precipitate of light flocks appears. The precipitate is centrifugated 15 minutes at 3,500 r/min. The acetone is decanted and used for the determination of choline and free acetylcholine. (It is kept cooled with solid carbon dioxide until worked up. A rather large vessel must be used as carbonic acid is very soluble in cold acetone and causes foaming when the temperature is allowed to rise.) To remove remaining free choline from the precipitate it is not sufficient simply to wash with acetone; the precipitate, which after centrifugation is a sticky mass, must be dissolved in 0.5—1 ml

of distilled water and again precipitated with 80 ml of acetone. This re-precipitation is made twice. The purified precipitate is treated with NaOD and acetylated as described on page 106.

As shown by experiment 1, the amounts of disturbing choline compounds are about 1,500 times as large as the amount of choline combined in the precursor. 97 p. c. of these impurities are removed by the first acetone fraction, about 2.9 p. c. in the second acetone fraction, and the remaining 0.1 p. c. in the third acetone fraction. The fourth acetone fraction contains practically no choline compounds which may be acetylated. In correspondence to these data we find that the amount of choline in the precipitate is constant after three precipitations. The third precipitate — and the following ones — contain only precursor choline. This is shown by the following facts: 1:o if the extract is heated in order to destroy the precursor before the treatment with acetone, the third precipitate contains no choline. 2:o if the precipitate is dissolved in water and precipitated with barium acetate, the barium precipitate contains the same amount of choline as the third acetone precipitate. 3:o if the acetone precipitate is dissolved in water, acidified and heated, and then precipitated with barium acetate, the precipitate contains no choline.

Experiment 1.

108 grammes of skeletal muscle (hind limb) of ranae temporariae were frozen with ether and solid carbon dioxide and extracted with ethyl alcohol. The extract was concentrated, washed with ether, dissolved in alcohol and again concentrated as described on page 105. The extract was then dissolved in distilled water and divided into 8 equal portions.

a) 5 portions were used for determining the amounts of choline in the various acetone fractions. They were precipitated 1—5 times respectively with acetone. The amounts of choline in the acetone soluble fraction and in the precipitate were determined as described on page 105. All amounts are expressed as γ of acetylcholine chloride per gram of tissue.

number of precipitations	precipitate	soluble
1	5.6	163
2	0.29	5.2
3	0.115	0.16
4	0.13	0
5	0.11	0

b) In order to split the precursor, one portion was made alkaline and heated for a couple of minutes, cooled, neutralized, and three

times precipitated with acetone. The amount of choline in the precipitate was determined and found to be less than 0.02 γ/gram as acetylcholine chloride.

c) 2 portions were used to study the precipitations with barium salts. Both portions were precipitated with acetone three times. The acetone precipitates were dissolved in about 10 ml of water. One of the portions was made alkaline and heated for a couple of minutes and then cooled and neutralized. Then both portions were precipitated with 1 ml of 25 p. c. barium acetate and centrifuged. The precipitate was washed twice with diluted barium acetate and centrifugated. The precipitate was washed twice with diluted barium acetate solution, and then it was acetylated according to the method on page 106.

The portion which had been heated contained less than 0.02 γ/-gram. The other portion contained 0.105 γ/gram.

II b. Separation of Free Acetylcholine and Choline from other Choline Compounds.

The amounts of free acetylcholine and choline are determined in the first acetone soluble fraction. As seen from experiment 1, practically all choline compounds appear in this fraction.

As shown by experiments 3 and 4, considerable amounts of acetylcholine as well as choline are lost if the acetone is evaporated to dryness. To evade losses, 5—10 ml of distilled water and 2 drops of concentrated acetic acid are added to the acetone, which is then evaporated 30° and at reduced pressure until all acetone is gone. This is controlled by a vacumeter; when only water is left, the pressure is rather abruptly lowered from about 90 to 15 mm Hg. The remaining watery solution is neutralized and its contents of free acetylcholine are biologically titrated.

The same watery solution is also used for the determination of choline. As mentioned above, the acetone soluble fraction contains not only free acetylcholine and choline but also other choline compounds which may form acetylcholine at acetylation. Part of these impurities have already been removed by washings with ether. If the impurities are not further removed, the amount of choline will be 50—100 p. c. too high. Therefore, the watery solution obtained after evaporation of the acetone is shaken three times with about 50 ml of petrol ether (B. p. 33°—45°). The petrol ether is drawn off with a suction pump. Petrol ether remaining in watery solution is then removed by evaporation. Part of the watery solution is then acetylated according to the description on page 106.

With regard to the determination of choline we have modified some details which implied risks for losses, but as a matter of principle the method described in this paper follows the usual lines. All substances which are soluble in alcohol, water, and acetone, but insoluble in petrol ether and ethyl ether, and which form acetylcholine at acetylation are determined as "choline". Some pharmacological experiments, which will be published later, seem to indicate that all this "choline" is not preformed in the tissues.

Experiment 2.

4 rabbits hearts, weighing 22 grammes, were extracted with ethyl alcohol, the extract was concentrated, washed with ether, dissolved in alcohol and again evaporated, and then precipitated with acetone according to the descriptions. 10 ml of water were added to the acetone soluble fraction; the acetone was evaporated. Of the remaining 10 ml of distilled water, 0.5 ml were taken out for acetylation. The rest of the solution was washed several times with 40 ml of petrol ether. After 2, 4, 6, and 8 washings a sample of 0.5 ml was taken out for acetylation. The amounts of choline found are expressed as γ/gram of acetylcholine chloride.

before washings	29.2 γ
after 2 »	18.0
» 4 »	18.8
» 6 »	16.0
» 8 »	17.6

III. Acetylation.

In the method described in this paper the precursor is broken down to choline and then acetylated to acetylcholine. Transformation of choline to its considerably more active acetyl ester has earlier been used for the determination of choline in tissue extracts. The principle was first used by Reid Hunt (1915); his method has since then been used with several minor modifications.

Organic chemists have of old used acetylchloride as well as acetic anhydride for the acetylation of several organic compounds. Both these substances have also been used for the acetylation of choline. LeHeux (1925) compared the acetylation with acetic anhydride and acetylchloride; he found considerably larger amounts of acetylcholine with acetic anhydride. The experiments

were, however, made on tissue extracts and not on pure choline. As seen from experiment 6, the anhydride gives a complete acetylation of choline. Acetylation with the anhydride can be made in an open vessel while boiling in glass bombs is necessary when acetylchloride is used. We have therefore used the more convenient anhydride method.

At the determination of tissue choline according to earlier methods, the extracts to be acetylated have as a rule contained 100 γ or more of choline chloride. Nevertheless, the losses may be exceedingly great (see LeHeux, 1925). As the amount of choline combined as acetylcholine precursor in the tissues is 1,000 times less than the amount of free choline, we must have a method, which admits the acetylation of far smaller amounts of choline. If the methods hitherto used would be applied on these small amounts, the losses would be approximately 100 p. c. We therefore had to examine each separate operation with regard to any measure which might be taken in order to avoid losses.

At earlier methods acetylation with acetic anhydride was assumed to be impossible in the presence of water. The tissue extracts have therefore been evaporated to absolute dryness. Roman has emphazised (1930) that choline is to some extent volatile, and when a solution is evaporated some choline disappears. Although Roman has been cited by some authors his findings have not been considered at the determination of tissue choline. As shown by experiment 3, the losses are moderate when larger amounts of choline are evaporated, but more considerable when the solutions are more dilute. The losses are greatest when a watery solution is evaporated and smaller when alcohol or acetone solutions are evaporated. When the solutions are evaporated to dryness the losses are considerable, but when a little amount of water is left, *e. g.* 0.5 ml, there are only moderate losses. This compelled us to examine if this small amount of water could be present at the acetylation (experiment 6). As shown, the addition of water does not render the acetylation incomplete if the concentration of water amounts to less than one tenth of the amount of acetic anhydride. We therefore now add at least 10 vol. of acetic acid for every vol. of watery solution. Thus the losses due to evaporation to dryness were avoided.

For how long time must choline be treated with acetic anhydride? At earlier methods one has heated the acetylation mixture on boiling water bath for 1—2 hours or boiled it in a retort with

reflux cooler for one hour. We found that the acetylation of these small amounts of acetylcholine is complete in less than 10 minutes at 100° C.

Another operation which may cause losses is the removing of the excess anhydride after acetylation. The anhydride itself and also sodium acetate formed at its neutralization disturbs the biological titration of acetylcholine on *m. rectus* as it causes shortening of the muscle. In experiments on 10 *m. recti* we found that 0.2—0.4 p. c. of sodium acetate in the Ringer solution caused a shortening; in one case we found a shortening at 0.1 p. c. sodium acetate. Smaller amounts, which do not cause any contraction themselves, had no influence on the biological titration of acetylcholine. From these experiments is seen that the excess of acetic anhydride must be very carefully removed. This has earlier been done by evaporating the anhydride to absolute dryness. As seen from experiment 4, greatest part of the acetylcholine is lost at this operation. Just as choline, acetylcholine solutions cannot be evaporated to dryness without considerable losses, whether the solvent is water, alcohol, acetone, or acetic anhydride (experiment 5). We therefore tried to remove the excess anhydride through washings with ether, which readily dissolves the anhydride. If no water is present at this operation, the ether will remove the acetylcholine as well as the anhydride. If losses should be avoided, the proportion water to anhydride must not be less than 5 (experiment 7). To avoid greater dilution of the acetylcholine solution, we therefore evaporate greatest part of the anhydride excess until only about 1 ml is left. Then 10 ml of water are added, mixed with the anhydride, and the mixture is shaken 10 times with about 40 ml of ethyl ether, which is drawn off with a suction pump after separation. This water solution of acetylcholine is neutral to litmus.

The accuracy of this acetylation method is satisfactory. It has been tested on solutions containing known amounts of choline, ranging from 10 γ to 0.3 γ as choline chloride (experiment 8). When the amount of choline to be acetylated is 1 γ or more, there are practically no losses. Even when 0.3 γ choline chloride is acetylated there is a loss of only 10 %.

Experiment 3. (Loss of choline at evaporation of various solvents).

To study the losses of choline at evaporation we made solutions containing 100 γ or 10 γ of choline chloride in 25 ml water, 25 ml of

absolute alcohol, or 25 ml of acetone. In some cases water was added. The solutions were evaporated at 30°, and at reduced pressure. In some cases the evaporation was carried on till absolute dryness, in other cases until 5 or 0.5 ml of water remained. The solutions were then acetylated according to the description.

	added amount	refound	loss in %
a. *Water solution*			
1) evaporated to dryness	100 γ	69	31
	10	3.7	63
2) evaporated to 0.5 ml.....	100	96	4
	10	8.8	12
	10	9.3	7
b. *Alcohol solution*			
1) evaporated to dryness	100	76	24
	10	4.5	55
2) 10 ml water added, evaporated to 5 ml	100	98	2
3) 2 ml water added, evaporated to 0.5 ml	10	9.5	5
c. *Acetone solution*			
1) evaporated to dryness	100	98	2
	10	7.4	26
2) 5 ml water added, evaporated to about 5 ml	100	104	—
3) 2 ml water added, evaporated to 0.5 ml	10	9.4	6

Experiment 4. (Loss of acetylcholine at evaporation of acetic anhydride.)

0.5 ml distilled water, containing 25 γ of acetylcholine chloride, and 9.5 ml acetic anhydride were evaporated

1) *to dryness at 50° C* and at reduced pressure: *refound* 11 γ, loss 56 p. c.
2) *to dryness at 80° C* and at reduced pressure, *refound* 0.
3) *to 2 ml volume at 80° C* and at reduced pressure. 10 ml distilled water was added and the micture was washed 10 times with 40 ml of ether, which was drawn off with a suction pump. Ether remaining in watery solution was evaporated. Reaction was controlled.

Refound: in 3 tests: 23.6, 24.8, 25.4 γ acetycholine chloride.

Experiment 5. (Loss of acetylcholine at evaporation of various solvents.)

To study the loss of acetylcholine at evaporation we made solutions containing 10 γ of acetylcholine chloride in 25 ml of water, absolute alcohol or waterfree acetone and in mixtures of 25 ml alcohol with

10 ml water and 25 ml acetone with 5 ml of water. 3 drops of concentrated acetic acid were added to all solutions. The solutions were evaporated at 30° C and at reduced pressure. In some cases the evaporation was carried out till complete dryness; in other cases it was finished when a certain amount of water was left. The evaporation vessel was rinsed with Ringer solution. The amount of acetylcholine chloride was determined on eserinized m. rectus of frog.

	refound	loss in p. c.
a) *Water solution*		
1) evaporated to dryness	1.7 γ	83
2) evaporated to 0.5—1 ml	9.8 γ	
	9.5 γ	
	9.4 γ	
b) *Alcohol solution*		
1) evaporated to dryness	0.35 γ	97
2) 10 ml water, evaporated to about 5 ml	9.7 γ	
	9.7 γ	
	9.3 γ	
c) *Acetone solution*		
1) evaporated to dryness	5.7 γ	43
2) 5 ml water added evaporated to about 5 ml	10.4 γ	
	9.6 γ	
	9.5 γ	

Experiment 6. (How much water can be present at acetylation with acetic anhydride?)

10 γ of choline bromide dissolved in 0.5—2.5 ml water and acetic anhydride as to make a total volume of 10 ml were transferred to test tubes. These were heated on boiling waterbath for 5, 20, 60, or 120 minutes. The volume was reduced to 2 ml through evaporation at 80° C and at reduced pressure. 10 ml water was added and the mixture of water and acetic anhydride was washed with 40 ml ether 10 times. Ether remaining in watery solution was removed by aeration. The amount of acetylcholine was titrated on m. rectus.

amount of water	amount of acetic anhydride	γ of choline bromide refound after heating for 5'	20'	60'	120'
0.5	9.5	9.8	9.8	10.0	9.7
1.0	9.0	7.3	9.6	9.8	10.1
1.5	8.5	3.4	5.9	9.8	.
2.0	8.0	0.6	0.8	1.4	7.2
2.5	7.5	0	0	0	.

Experiment 7. (Loss of acetylcholine at the washing of mixtures of water and acetic anhydride with ether.)

To 10 γ of acetylcholine chloride dissolved in 10 ml of water varying amounts of acetic anhydride were added. The mixtures were shaken 10 times with 40 ml of ether, which was drawn off. Ether remaining in the watery solution was removed by aeration at reduced pressure. The amounts of acetylcholine chloride were determined on eserinized m. rectus of drog.

ml acetic anhydride added	γ acetylcholine chloride refound		loss in p. c.
10	3.2,	3.8	65
5	5.9,	6.4	38
3	8.3,	9.1	13
2	9.3,	9.6	5
1	9.5,	10.2	—
0	9.5,	9.8	—

Experiment 8. (Accuracy of the acetylation method.)

To test the accuracy of the acetylation method proposed in this paper we made series of determinations on known amounts of choline chloride, varying from 10 to 0.3 γ. Every series consisted of 12 determinations. The acetylcholine was dissolved in 0.5 m. water, 9.5 ml of acetic anhydride was added and the mixture was acetylated and treated in full accordance with the description above. In series IV the 10 ml of watery acetylcholine solution obtained after acetylation was so dilute that titrations on m. rectus was not possible. It was, therefore, before titration concentrated to 2 ml by evaporation at 30° C and at reduced pressure. The amounts of acetylcholine were titrated on eserinized m. rectus of frog. In series IV the amount of acetylcholine only sufficed for 2 titrations; in the other series 4 determinations were made at every determination.

Series I. Added amount: 10 γ choline chloride = 12.8 γ acetylcholine chloride. Refound 12.6 $\pm$ 0.17; Standard deviation 0.61=4.8 %.

Series II. Added amount: 3.5 γ choline chloride = 3.84 γ acetylcholine chloride. Refound: 3.5 $\pm$ 0.075; Standard deviation 0.26=6.8 %.

Series III. Added amount: 1.0 γ choline chloride = 1.28 γ acetylcholine chloride. Refound 1.18 $\pm$ 0.027; Standard deviation 0.094 = = 7.6 %.

Series IV. Added amount 0.3 γ choline chloride = 0.38 γ acetylcholine chloride. Refound 0.331 $\pm$ 0.011; Standard deviation 0.0038 = = 10.8 %.

Biological Titration of Acetylcholine.

For titration of acetylcholine many methods have been designed. CHANG and GADDUM (1933) and GADDUM (1936) have

reviewed and compared the different methods. They consider the eserinized m. rectus of frog the most reliable test object. The frog's heart and the intestine of rabbit or mouse are sensitive preparations but rather unspecific and readily influenced by other agents present in tissue extracts. According to our experience the very sensitive leech preparation works more slowly and is considerably less accurate than the frog muscle. We have therefore used the eserinized m. rectus. The best preparation is obtained from *rana esculenta*, but the war has compelled us to use Swedish temporaria males. The preparation is rather specific and sensitive enough for our purpose. Addition of 0.01—0.02 γ acetylcholine chloride is sufficient to provoke a proper shortening.

The *m. rectus* preparation gives more accurate values than other preparations. We made 20 determinations on a known amount of acetylcholine. Every determination consisted of one addition of a standard solution and one addition of test solution. The standard deviation of this series was found to be 8.0 p. c.; the maximal deviation found was 19 p. c. At the determination of tissue acetylcholine we always made 4 titrations. In these cases the accurary at the titration will be 4 p. c. In series of titrations on frog's heart and rabbit's intestine made by LeHeux (1925) the standard deviations were 13.6 and 11.1 p. c. respectively.

The muscles were prepared in the usual way and for 60 minutes laid in oxygenated Ringer solution, to which had been added eserine sulfate to a final concentration of 1 : 200000. Although this concentration of eserine is sufficient completely to inhibit the esterase of frog muscle *in vitro*, it does not give the maximal sensitiveness to acetylcholine. Greater amounts of eserine will, however, often impede the relaxation of the muscles after the acetylcholine has been washed out. The shortening of the muscles was registered with a light isotonic lever. — If the acetylcholine to be tested is not dissolved in salt solution but in water, the volume added to the bath should not exceed 10 p. c. of the bath volume. If greater amounts were added we often found that the contraction developed was greater than the amount of acetylcholine accounted for, but at the same time the preparation was destroyed and its sensitiveness to following acetylcholine additions was decreased. If the amount of acetylcholine present in the test solution was not enough to give a measurable shortening when added in these proportions, the solution was concentrated by evaporation at 30° C and at reduced pressure.

The relation between acetylcholine concentration and the shortening of the muscle is not linear. It follows the law of LANGMUIR-HITCHCOCK (CLARK, 1936): $k \cdot y = \frac{x}{a - x}$; k is a constant, y is the concentration of acetylcholine provoking the effect x; a is the maximal effect which can be provoked by acetylcholine. All calculations ought to be made according to this formula. It is, however, necessary to emphazise that sometimes the small effects do not follow the LANGMUIR-HITCHCOCK law but require considerably greater concentrations of acetylcholine. This is due to infavourable experimental conditions as too heavy levers or to great friction between paper and lever. It is therefore advisable always to use a concentration of standard, which gives about the same effect as the test.

Accuracy of the Method.

a. *with regard to the determination of the precursor.*

As mentioned above the biological titration implies an error of 4 p. c. — as measured by the standard deviation — if 4 titrations are made at every determination. Acetylation + titration has an error of about 6 p. c. The determination of the precursor consists of many other operations which may cause errors. To measure these errors we made an alcoholic extract of ox spleen according to the description on page 104. After filtration and centrifugation the extract was divided into 20 equal parts. The amounts of precursor in 10 of these parts were determined according to the method proposed in this paper, and were found to contain:

2.4, 2.3, 2.15, 2.15, 2.1, 2.05, 1.95, 1.85, 1.8, and 1.7 γ of precursor expressed as acetylcholine chlorid. The mean value was 2.05 ± 0.07, standard deviation $0.22 = 10.7$ p. c.

To be able to judge any value obtained with this method we must know not only the standard deviation but also the aggregate losses of the precursor, which may occur at every treatment of the extract. As shown by experiment 8, acetylation only may cause a loss of 5—10 p. c. To determine the total losses portions of the same extract, as had been used for the series above, were evaporated, washed with ether, re-dissolved in alcohol, evaporated and finally precipitated 3 times with acetone according to the method described above. According to the series above, the acetone insoluble fraction contains 2.05 γ of precursor calculated as acetylcholine chloride. The precursor was dissolved in 75 p. c.

ethyl alcohol, which corresponds to the concentration of alcohol solution was treated just as a tissue extract. Its contents of precursor were determined. The series gave the following data: Mean value 1. 0 ± 0.05, Standard deviation $0.17 = 9.2$ p. c. As seen, we only refound 1.80 γ of the added amount 2.05 γ. The operations have caused a loss of $0.25 \pm \sqrt{0.07^2 + 0.05^2}$ *i. e.* 12 ± 4 p. c. of the added amount. This value does not include losses and errors caused by inadequate extraction.

b. *with regard to free acetylcholine.*

200 grammes of rabbit's skeletal muscle were extracted with alcohol according to the description. The extract was made alkaline to phenolphtalein and left standing at room temperature for 5 hours. Thereby free acetylcholine as well as precursor were destroyed. The extract was then acidified with acetic and its volume made up to 600 ml. 50 γ acetylcholine chloride were added. The extract was divided into 10 equal parts. In each part the free acetylcholine was determined according to the method proposed in this paper. One portion was lost.

The following series was found:

4.9, 4.8, 4.6, 4.3, 4.3, 4.2, 4.1, 3.9, 3.8;

Mean value: 4.36 ± 0.12. Standard deviation $0.37 = 7.4$ p. c.

As seen, there is a loss of 13 ± 2.4 per cent of the added amount, i. e. practically the same value as found in the series of the precursor.

Summary.

A method for determination of acetylcholine precursor, free acetylcholine, and choline in tissues is described.

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The Action of Ergotamine on the Chemical and Mechanical Reflexes from the Carotid Sinus Region.

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ROTHLIN showed in 1923 that the fall in blood pressure caused by stimulation of the depressor nerves in the rabbit was greatly diminished by ergotamine. HEYMANS and REGNIERS (1929) studied the effect of ergotamine on the sinus reflexes and found that while the vasomotor reflexes from the sinus region were suppressed by ergotamine (0.25 mg ergotamine tartrate pr kg) in the dog, the cardio-inhibitory reflexes persisted or were even temporarily reinforced. Using smaller doses, 0.1—0.15 mg/kg in the cat, WRIGHT (1930) was able to state that the vasomotor reflexes from the sinus were abolished, though the dose of ergotamine was not sufficient to suppress the effect of injected adrenaline or else block the peripheral vasomotor mechanism. On the other hand central asphyxia was still active in producing a rise in blood-pressure. He therefore drew the conclusion that ergotamine inhibited the central transmission of impulses on the afferent side of the vasomotor centre. HEYMANS, REGNIERS and BOUCKAERT (1930), in a continuation of their previous study, showed that when 0.25 mg ergotamine tartrate per kg was injected into a dog, the head of which was perfused by another dog, occlusion and opening of the common carotids leading to the perfused head had no effect on the blood pressure of the rest of the body. The animal was in this case insensitive even to injected adrenaline, however. On the other hand a similar dose of ergotamine injected into the

perfuser, thus reaching the centres of the isolated perfused head of the other dog, did not suppress the effect of clamping the carotids to the perfused head, though the rise in blood pressure was slower and somewhat reduced. 1 mg of ergotamine tartrate injected into the third ventricle was effective, however, in this respect. The authors concluded from these experiments that the effect was chiefly peripheral, though a central action could also be demonstrated which they located to the vasomotor centre.

In man, NORDENFELT (1941) obtained evidence for a suppressing action of ergotamine (0.5 mg Gynergen intravenously) on the buffer nerve mechanism. Thus the injection caused an average increase of 13.0 $\pm$ 2.3 mm Hg in the general blood pressure when the patients were in recumbent position. On raising to standing position the fall in blood pressure was greater than normally, especially in patients with orthostatic anaemia, indicating that the regulatory mechanism was less efficient.

The establishment of the fact that small doses of ergotamine may completely inhibit the rise of blood pressure caused by occlusion of the carotids raises the following questions:

1) Does ergotamine also affect the reflexes elicited by stimulation of the chemical receptors of the sinus region, and especially those connected with the vasomotor system.

2) Does ergotamine exercise a direct effect on the receptors.

In order to answer the first question we have studied the effect of ergotamine on respiration and blood pressure during hypoxia and also the action of the drug on the blood pressure raising effect of cyanide which is known to act chiefly on the chemoreceptors.

A direct answer to the second question could be obtained by recording the action potentials from the sinus and glomus receptors in the nerve of Hering under experimental conditions involving stimulation of the different types of receptors

Methods.

In the first part of this investigation the effect of ergotamine tartrate[1] was studied on the hyperpnoea elicited by oxygen want and on the rise in blood pressure obtained by clamping the common carotids.

Cats and rabbits were used as experimental animals and anesthetized with 0.06 g chloralose (cats) or 1.8 g urethane (rabbits) per kg body weight intravenously. Respiration was measured quantitatively by means of the body plethysmograph previously described by EULER

[1] Sandoz A.-G., Basel, kindly supplied us with "Gynergen".

and LILJESTRAND (1936). Arterial blood pressure was recorded with a Hg-manometer from the femoral artery (cats) or from the left carotid (rabbits). Injections were made through the femoral vein. The animals were allowed to breathe spontaneously through Müller valves.

In order to control the activity of the pressoreceptors both carotids could be clamped simultaneously by means of small clips which could be handled from outside the plethysmograph.

The chemoreceptors were stimulated by means of gas mixtures containing 7—15 % oxygen in nitrogen, kept in bags which could be attached to the inspiratory side of the Müller valve.

The second part of the investigation included the recording of action potentials from the sinus nerve and the effect of intravenously injected ergotamine thereupon during various experimental conditions involving stimulation of the stretch receptors and chemoreceptors. The electroneurograms were obtained from cats, anesthetized as above, according to the method described by ZOTTERMAN (1935)[1]. Blood pressure was recorded by means of an elastic manometer connected with the femoral artery.

The cats either respired spontaneously through a tracheal cannula or were artificially ventilated with a Starling respiratory pump, to which rubber bags containing the desired gas or gas mixture could be attached

Injections were made through the femoral vein.

Results.

A. Effect of Ergotamine on the Carotid Occlusion Test and on the Respiratory Reflex Induced by Oxygen Want.

The effect of ergotamine on the mechanically induced sinus reflex acting on the blood pressure, and on the chemical reflex affecting respiration, is shown by the following experiment.

Cat, 3.45 kg, chloralose. Mechanical reflex tested by clamping both carotids; chemical respiratory reflex tested by spontaneous respiration of 6.8 % and 10.5 % O_2 in N_2 for about 2 min. Ergotamine tartrate 0.1 mg/kg intravenously.

	Before ergotamine	After ergotamine
Increase in B.P. caused by clamping both carotids, mm Hg.		
a) oxygen	58	5
b) air	70	26
Respiratory volume l per minute before the hypoxia	0.88	0.82
Respiratory volume l per minute during hypoxia	1.66	1.65
Per cent increase in respiratory volume	89	101

[1] Dr. Y. ZOTTERMAN kindly placed his recording set at our disposal.

It is evident from this experiment that clamping the carotids gives a much smaller effect after ergotamine, even in small doses, than before, whereas the chemical respiratory reflex shows no sign of being weakened. On the other hand the "resting" respiratory volume gradually diminished, probably owing to central depression as in the experiments by EULER (1938). A peripheral blocking did not occur as shown by the fact that adrenaline was still effective in a dose of 40 μg injected intravenously.

In another experiment where the rise of blood pressure, following bilateral clamping of the carotids, amounted to between 30—40 mm Hg, a dose of only 0.01 mg ergotamine tartrate per kg reduced the rise to 8 mm Hg. In the same animal the increased ventilation following hypoxia was not influenced, the increase amounting to about 60 %. In two other cats and in the rabbits the results were essentially similar, the occlusion pressor effect being greatly reduced after doses of 0.1 mg/kg.

B. Effect of Ergotamine on the Chemical Vasomotor Reflex Elicited by Oxygen Want and Cyanide.

A reflex rise of blood pressure as a result of stimulation of chemoreceptors was first demonstrated for synaptotropic substances and cyanides by HEYMANS, BOUCKAERT, EULER and DAUTREBANDE (1932). The effect of oxygen lack in this respect was later shown by WINDER, BERNTHAL and WEEKS (1938) and BJURSTEDT and EULER (1943). Though the effect is hardly noticed under ordinary experimental conditions it may become spectacular under certain circumstances. Thus EULER and LILJESTRAND (1943) recently showed that occlusion of the common carotids gives a markedly increased effect when the animal is made to breathe a gas mixture poor in oxygen. The carotid occlusion in this case will cause hypoxia in the sinus region besides the lowering of intrasinusal pressure, both effects co-operating to increase the blood pressure.

As stated above a certain rise in blood pressure often follows on clamping the carotids in the cat even after ergotamine. This is illustrated by Fig. 1 D and E and also by Fig. 2 and 4. It might be argued that this effect is due to an incomplete inhibition of the sinus baroceptor reflex by the small dose of ergotamine used. This is not the case, however, since it could be shown that the effect completely disappears (but for the mechanical haemo-

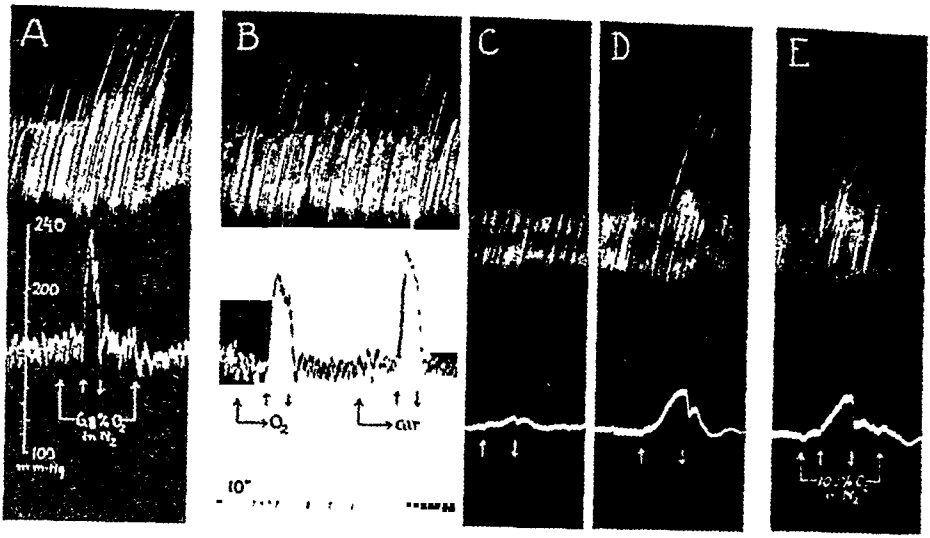

Fig. 1. Cat, 3.45 kg, chloralose. Upper curve respiration, lower curve blood pressure.

A. Increased respiration on administering 6.8 % oxygen in nitrogen. Carotid occlusion during hypoxia ↑↓.

B. Occlusion test during oxygen and air breathing.

C. Occlusion test during oxygen [illegible] after 0.1 mg ergotamine tartrate i.v. per kg.

D. Same during air breathing.

E. Same during breathing of 10.5 % oxygen in nitrogen.

dynamic rise) when the animal is allowed to breathe oxygen (Fig. 1 C and 2). In fact, the occlusion pressor effect after ergotamine may or may not appear during ordinary breathing of air, depending on the degree of hypoxic stimulation caused by the clamping of the carotids, which in its turn depends on the intensity of ventilation and the general blood pressure level. If absent, the occlusion effect then may be called forth by administration of a gas mixture poorer in oxygen. The explanation of the occlusion effect along these lines also gains support by the fact that the pressure response closely parallels the chemoreflex stimulation of the respiration, as shown in the figures.

After bilateral vagotomy the chemical reflexes on the blood pressure and on the respiration elicited by occlusion hypoxia are still present. In the experiment, to which Fig. 2 refers, a specially marked effect was obtained by letting the animal breathe 15 % oxygen in nitrogen. No effect on the blood pressure resulted from carotid occlusion during oxygen breathing.

When in addition both sinus regions had been denervated the respiratory effect of carotid clamping disappeared (Fig. 3 A).

Still some effect on the blood pressure remained, however, which must be regarded as centrally induced. The effect disappeared when artificial respiration with air was given to an extent, sufficient to raise the blood pressure to the pre-denervation level. As a matter of fact the blood pressure could be changed at will from anything between 50 and 130 mm Hg in the sinus denervated animal simply by adjusting the artificial ventilation or by administering gas mixtures more or less rich in oxygen during spontaneous breathing. It is not quite clear, however, why central hypoxia due to carotid occlusion should raise the

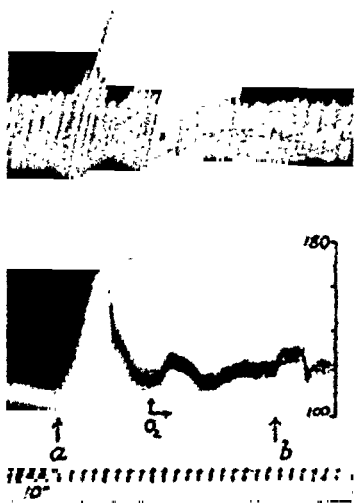

Fig. 2. Same experiment as in Fig. 1. After ergotamine tartrate 0.1 mg/kg and bilateral vagotomy. Carotid occlusion during a) hypoxia (15 % O_2 in N_2) and b) oxygen breathing. Note parallel effects on ventilation.

general blood pressure after sinus denervation whereas general oxygen lack causes a fall. Possibly the cause of this difference is that the degree of oxygen want and to the accumulation of acid metabolites in the centres do not run parallel.

Like oxygen want cyanides are capable of inducing a reflex stimulation of respiration (HEYMANS, BOUCKAERT and DAUTREBANDE, 1931) and of the vasomotor system (HEYMANS, BOUCKAERT, EULER and DAUTREBANDE, 1932).

The administration of 0.5 mg potassium cyanide intravenously produced, even after ergotamine, a marked stimulating effect on respiration and blood pressure, showing that the chemoreflex system was still functioning well (Fig. 4). As a control the injection was repeated after complete denervation of the reflex regions.

During spontaneous respiration the effect was inhibition of respiration and a fall in blood pressure (Fig. 3 A). When respiration was kept constant artificially no change occurred in blood pressure (Fig. 3 B) which makes it probable that the fall in the former case was due to the inhibition of the respiration, caused by a direct central effect of the cyanide injection.

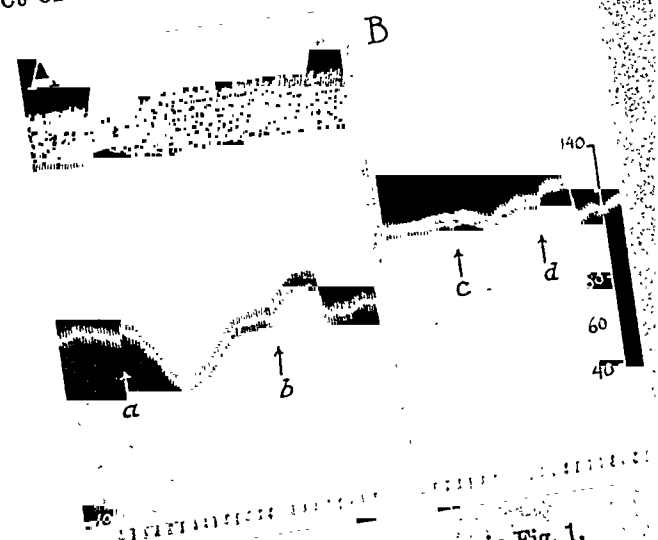

Fig. 3. Same experiment as in Fig. 1.

Bilateral vagotomy and denervation of both sinus regions.
A. Spontaneous respiration. a) 0.5 mg KCN i.v. b) carotid occlusion.
B. Artificial respiration. c) 0.5 mg KCN i.v. d) carotid occlusion.

Fig. 4 reveals an action of cyanide on the chemoreceptor reflex mechanism which does not seem to have attracted notice earlier. After the dose of cyanide employed, the occlusion hypoxia reaction, both on blood pressure and respiration, was definitely diminished for a while but later recovered. This subacute effect would seem to be the result either of a temporary injury to the central transmission system or the peripheral glomus mechanism which is assumed to involve a synaptic transmission (EULER, LILJESTRAND and ZOTTERMAN, 1941). On the other hand the vasomotor and respiratory centres show no sign of depression, judging from the respiration and blood pressure.

In Fig. 4 b the stimulating effect of a small dose of nicotine is also illustrated.

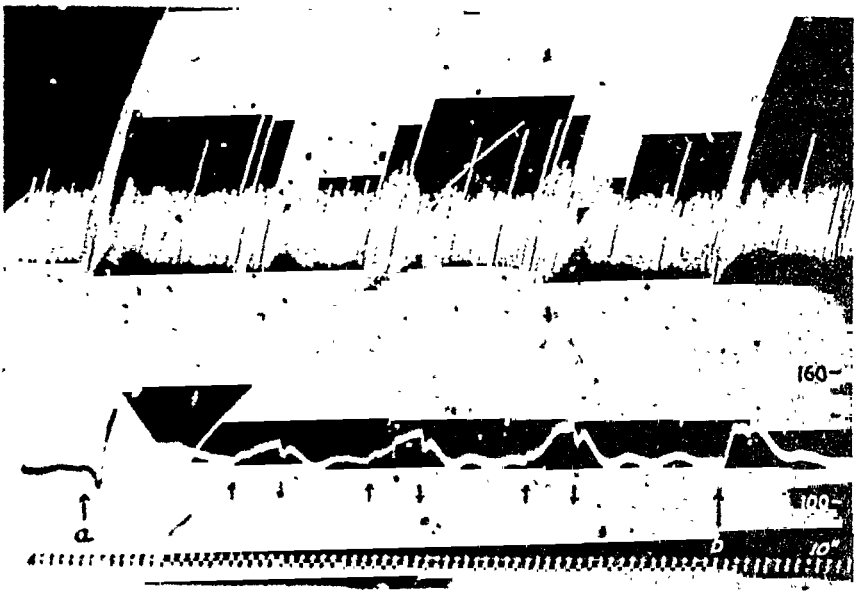

Fig. 4. Same experiment as in Fig. 1. 55 min. after 0.1 mg ergotamine tartrate per kg. a) 0.5 mg KCN i.v. ↑↓ carotid occlusion. b) 0.05 mg nicotine tartrate i.v.

C. Effect of Ergotamine on the Action Potentials in the Sinus Nerve.

The two types of receptors in the sinus region, those responding to mechanical stimuli in the sinus proper and the chemoreceptors in the carotid body give rise to different types of action potentials in the sinus nerve as demonstrated by Heymans and Rijlant (1933), Bogue and Stella (1934, 1935), Zotterman (1935), and more recently by Euler, Liljestrand and Zotterman (1939). The doubts expressed by Schmidt and Comroe (1940) as to whether the chemical potentials are actually set up in the chemoreceptors of the carotid body have clearly been shown to lack an evidential basis (Euler and Zotterman 1942).

In the present investigation it was found that the large pressure "spikes" were not reduced in frequency or else affected as a result of injections of ergotamine (Fig. 5 a, b), even in doses of 0.25 mg/kg, although the blood pressure response to lowered intra-carotid pressure was greatly reduced as controlled by manometer readings. Ergotamine in itself caused a temporary elevation in general blood pressure and this, as under normal conditions, gave rise to an increased amount of pressure impulses. Likewise the strong rise in blood pressure, caused by injection of 0.1 ml Veritol greatly increased the frequency of the pressor impulses (Fig. 5 d).

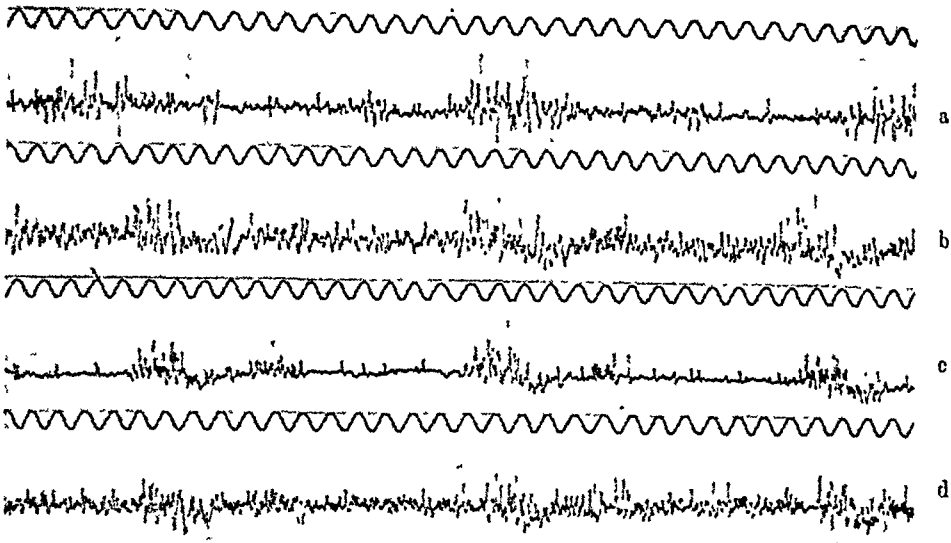

Fig. 5. Electroneurogram from the sinus nerve of a cat. Constant artificial respiration.

a) air, before ergotamine;
b) 7% oxygen in nitrogen, 7 min. after 0.1 mg ergotamine tartrate i.v. per kg.
c) air;
d) air, during rise of blood pressure after i.v. injection of 0.1 ml Veritol (2 mg β-(p-oxyphenyl)-isopropylmetylamine). Time marking $^1/_{50}$ sec.

The increase in the electrical discharge consisting of small potentials, produced by hypoxia, still persisted after administration of ergotamine (Fig. 5 b) even in doses of 0.5 mg per kg body weight, which was in accordance with expectation, since the chemical reflexes were left patent.

It follows from this that the inhibition of the sinus stretch reflex is not on the receptor system but must be sought for in the central transmission system.

Discussion.

The question as to the mechanism of action of ergotamine on the vasomotor effects elicited from the buffer nerve receptor regions seems to be intimately connected with the dose of ergotamine used. Whereas larger doses, sufficient to block the peripheral action of adrenaline or direct vasomotor stimulation, necessarily must strongly reduce the effect of carotid occlusion on the vagotomized animal, a small dose, found to inhibit the pressor reflex, must act in a different way. As to the minimal dose of ergotamine required to inhibit the blood pressure reflexes from the moderator nerves, it has been observed that even doses of 0.01—0.1 mg/kg

may effectively prevent the reflex in the cat or the dog (HEYMANS, REGNIERS and BOUCKAERT, WRIGHT). Our results are in accord with these findings. It is obvious that doses of this order are not capable of producing a peripheral blocking of the vasomotor system and it follows therefore that the effect must be located elsewhere. An action on the pressor receptors is rendered less probable already by the fact that reflex slowing on the heart may still be elicited by stimulation of the pressor receptors in the sinus (HEYMANS and REGNIERS). Our experiments have, indeed, directly demonstrated that the pressor impulses in the sinus nerve are patent after ergotamine.

The fact that glomus hypoxia, cyanides and central asphyxia still may produce a reflex rise in blood pressure, though hypotension as such in the sinus is ineffective after a dose of ergotamine of the order of 0.1 mg/kg, indicates that the block is not on the efferent side of the vasomotor centre. It thus seems necessary to assume an elective action on a special part of the central transmission system. Whether or not the action is located to some part of the vasomotor centre or to some synapse on the afferent side does not seem to be a case of easy decision. The interesting point is, however, that the reflex impulses elicited by chemical stimuli still find their way to cause a reflex rise in blood pressure, whereas the stretch reflex system is blocked, in spite of the fact that the nervous paths would be assumed to coincide at least partly.

Our experiments have shown that small doses of ergotamine are sufficient to prevent the quick phase in the rise of blood pressure following carotid occlusion. Using higher doses it was also possible, however, to reduce the chemical vasomotor reflex before any blocking effect on the action of injected adrenaline could be noticed. Thus even the chemical reflex system may be inhibited by ergotamine as to its vasomotor part, whereas the respiratory reflex paths still remained in function. If "occlusion hypoxia" is the cause of the remaining blood pressure rise after small doses of ergotamine also in the dog, the effect shown in Fig. 3 in the paper of HEYMANS, REGNIERS and BOUCKAERT (1930) would be due to a stimulation of the chemoreceptors acting on the vasomotor system. Thus the case might be that the stretch receptor reflex is suppressed, whereas the "chemical" reflex persists. The reason for the unusually strong appearance of this reflex, elicited by occlusion hypoxia after ergotamine, would then be sought in the absence of the normal moderator effect from the baroceptors.

The suppressing action of ergotamine in small doses on the moderator nerve mechanism has proved of great practical value in routine laboratory experiments when blood pressure active substances are tested. Sometimes the abolishment of the presso-regulating system will impose as a greatly increased sensitivity for the substances in question, and further, as an added advantage, the smoothing effect on the blood pressure curve is registered.

Summary.

Ergotamine in small doses (0.1 mg/kg) inhibits selectively the carotid sinus pressor reflex, elicited by lowering the intra carotid pressure, whereas the response to chemical stimuli, such as hypoxia, cyanides, or nicotine, still can be obtained, not only on respiration but also on the blood pressure.

If a pressor response to carotid occlusion still occurs after ergotamine, it disappears when oxygen is administered. The effect in the former case is then due to occlusion hypoxia which stimulates the chemoreceptors connected with the vasomotor system.

It could be shown that the abolishment of the sinus pressor reflex, following administration of ergotamine, is not due to an effect upon the receptors, since the action potentials set up in the sinus nerve by the intrasinusal pressure can still be elicited.

Treatment of the experimental animal with small doses of ergotamine affords a convenient way of suppressing the sinus and aortic presso-regulating system.

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Nachweis von Prontosil solubile in histologischen Gewebsschnitten mit Hilfe der Fluoreszenzmikroskopie.

Von

STURE HELANDER.

Eingegangen am 4. Mai 1944.

Der Nachweis eines Pharmakons in histologischen Schnitten, und zwar derart, dass die Lokalisation desselben nicht nur in den verschiedenen Organen und Geweben, sondern auch in der einzelnen Zelle unmittelbarer Wahrnehmung im Mikroskop zugänglich gemacht wird, besitzt unter vielen Gesichtspunkten ein gewisses Interesse. Mittels einer derartigen Methode können ja solche Probleme wie Wege der Resorption, Verteilung im Organismus und in den Zellen unter verschiedenen Bedingungen sowie Ausscheidung der betreffenden Substanz in Angriff genommen werden.

Bei histopharmakologischen Untersuchungen führt die gewöhnliche histologische Methodik aus leicht begreiflichen Gründen nicht zum Ziel.

Die in letzter Zeit weiter ausgebaute Altmannsche Gefriervakuumtechnik zur Fixierung von Organpräparaten (Gersh 1932, 1938, Hoerr 1936, F. Sjöstrand und T. Sjöstrand 1938, Simpson 1941 sowie F. Sjöstrand 1944) genügt indessen den Anforderungen, welche vernünftigerweise an eine derartige histopharmakologische Technik gestellt werden können.

Zur Darstellung von Pharmaka in Gewebsschnitten eignet sich besonders die Fluoreszenzmikroskopie, da sich bei dieser Eingriffe erübrigen, welche die Lokalisation der Substanz verändern könnten. Verf. (1943) hat in Kürze ein Verfahren angegeben, welches den Nachweis von u. a. Sulfathiazol in histologischen Schnitten mittels der Fluoreszenzmikroskopie gestattet. Dadurch ist es möglich geworden, u. a. die Resorption des Sulfa-

thiazols durch die Nasenschleimhaut zu verfolgen (HELANDER, RICHTNÉR, F. SJÖSTRAND, T. SJÖSTRAND 1943).

Bei weiteren Untersuchungen erwies sich Prontosil solubile als zu diesbezüglichen Studien hervorragend geeignet.

Früher hatte HACKMAN (1942) eine Methode beschrieben, dieses Medikament mit Hilfe seiner intensiv roten Eigenfarbe in Gewebsschnitten nachzuweisen. Die Präparate waren dabei in einem Äther-Kohlensäureschneegemisch gefroren und dann im Vakuumexsikkator über Phosphorsäureanhydrid entwässert worden, d. h. es war von Altmanns ursprünglichem Gefrier-Trockenverfahren Gebrauch gemacht worden.

Ferner wurde über eine Methode berichtet, eine Reihe von ungefärbten Verbindungen in Azofarbstoffe überzuführen.

Das fluoreszenzmikroskopische Verfahren ist aber wesentlich empfindlicher und auch in mehreren anderen Beziehungen vorteilhafter. Der Arbeitsgang ist dabei folgender: Unmittelbar nach der Tötung des mit Prontosil solubile behandelten Versuchstiers werden kleine Stücke derjenigen Organe und Gewebe welche man zu untersuchen wünscht, entnommen und in flüssiger Luft gefroren. Dann werden die Präparate im Kühlschrank bei — 40° C vakuumgetrocknet, wobei die Wasserdämpfe von Phosphorsäureanhydrid aufgenommen werden. Hierauf werden die Präparate in Paraffin eingebettet, geschnitten, trocken auf Objektträger gelegt und sind damit ohne Auslaugung des Paraffins zur mikroskopischen Untersuchung fertig. Bezüglich näherer Einzelheiten dieser Technik wird auf F. SJÖSTRAND (1944) verwiesen. Bei dieser äusserst schonenden Behandlung werden alle die Fehlerquellen vermieden, welche die zahlreichen Lösungsmittel der gewöhnlichen histologischen Technik mit sich bringen.

Im gewöhnlichen Licht findet man da, dass sich diejenigen Teile der Schnitte, welche grössere Mengen von Prontosil solubile enthalten, durch ihre rote Farbe von der schwach gelblichen Umgebung unterscheiden. Nähere Einzelheiten der Lokalisation lassen sich jedoch ohne Färbung der Präparate schwer feststellen.

Im Fluoreszenzmikroskop (Verf. hat Reicherts grossen Fluoreszenzapparat Lux U. V. angewendet) wird das Bild allerdings bei weitem reichhaltiger. Gegen die tiefblaue Farbe der Gewebe sticht nämlich das Prontosil solubile mit seiner intensiv scharlachroten Fluoreszenzfarbe äusserst scharf ab, und man entdeckt jetzt diese Substanz auch dort, wo sie in den einzelnen Zellen

in sehr kleinen Mengen vorkommt, und wo man im gewöhnlichen Licht nicht die geringste Spur derselben sehen konnte.

Dank der Eigenfluoreszenz der Gewebe bietet die Lokalisation keinerlei Schwierigkeiten, da die meisten Formelemente ebenso deutlich hervortreten, wie in gefärbten Präparaten.

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The Localization of Mineral Salts in Striated Muscle-Fibres.

By

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Received 8 May 1944.

Among the substances which build up the striated muscles, the adenylic acids, in view of their characteristic ultraviolet absorption spectrum, afford particularly good facilities for direct localization and determination of the concentration in the cell structure. The purin group in these adenylic acids has a well-defined absorption maximum at 2 600 Å. The other constituents of the molecule, namely the sugar and phosphoric acid, have no influence on the absorption spectrum. As macrochemical investigations have shown that about 95 per cent. of all the nucleotides in the resting striated muscles consist of adenylic acids, and that free purins or other substances with a similar absorption type occur merely in very minute amounts, a characteristic nucleotide absorption spectrum in resting muscles is an almost certain indication of the presence of adenylic acids.

The large amount of phosphoric acid contained in the adenylic acids might also be used for localization of those substances. Adenosin triphosphoric acid contains about 48 % phosphoric acid. On micro-incineration of a section of striated muscle, the adenylic acids will therefore yield large amounts of ash. It has previously been shown by the author that, as regards the polynucleotides (with about 25 % phosphoric acid) of the cell nucleus and cytoplasm, there is an evident correlation between ultraviolet absorption and amount of ash (ENGSTRÖM, 1943). The concentration of nucleic acids in the said cell structures varies considerably. For example, the nucleolus in the spinal ganglion cells

(type II) of *Lophius piscatorius*, according to HYDÉN (1943), has a concentration of $0.7 \cdot 10^{-11}$ mg/μ^3, whereas the nucleic acid concentration in the basal parts of the exocrine gland cells in the pancreas amounts to about $4 \cdot 10^{-11}$ mg per μ^3 (CASPERSSON, LANDSTRÖM-HYDÉN and AQUILONIUS, 1941). These last mentioned cell parts yield very large amounts of ash, but the ash left by the nucleolus in the spinal ganglion cells of *Lophus piscatorius* is also distinctly visible.

According to macrochemical investigations, striated mammalian muscles contain, on an average, about 0.1—0.2 % adenylic acids, which corresponds to $0.1—0.2 \cdot 10^{-11}$ mg/μ^3 (For references, see FENN, 1936). With ultraviolet absorption measurements, CASPERSSON and THORELL (1942) found, in the striated muscles of certain invertebrates, a percentage of adenylic nucleotides corresponding well with the macrochemically found amounts in mammals. *The ash left by the adenylic acids, on micro-incineration of a section in which they have not been liberated, should thus be visible even if the adenylic nucleotides are equably distributed over the whole muscle fibre.* Now, according to CASPERSSON and THORELL (1942), the adenylic acids are mainly concentrated in the isotropic segments. In those segments, therefore, the amount of adenylic acid per μ^3 will thus be considerably larger than the average value given above. Moreover, as the major part of the adenylic acids is found in the form of adenosin triphosphoric acid (with about 48 % phosphoric acid, as compared with about 25 % in the other nucleotides), it will be understood that the ash left by the selectively ultraviolet-absorbing segments in the striated muscle-fibres should be distinctly visible in a micro-incinerated preparation.

A number of investigators, on micro-incineration of striated muscles, found that the striated structure was well preserved in the ash (TSCHOPP, 1929, SCOTT, 1932/33, KRUSZYŃSKI, 1938, CAREY and ZEIT, 1939, LORETI, 1940, etc.) SCOTT, having made such an observation, on micro-incineration of the rectus muscles of cat, and, after comparing the micro-incinerated and the adjacent hematoxylin-stained section, maintained that the ash was derived from the anisotropic segments. KRUSZYŃSKI's investigations were made on material where the muscle partitions were somewhat higher than in normal mammalian musculature. He found that the ash consisted of short little rods, alternating with spaces almost free from ash, and contended that it was derived

from the myofibrils. After comparison between normal and contracted tissue, he expressed the view that the ash was yielded by the anisotropic segments. In satisfactory preparations, the said authors sometimes found in the ashfree area a fine streak of ash, which was interpreted as Z.

Thus, the methods adopted by the investigators who have resorted to the micro-incineration of striated muscles with a view to the localization of the ash are comparison between the micro-incinerated and the adjacent stained section and studies over contracted muscle tissue.

These methods, however, are by no means satisfactory, especially as regards muscles with very low partitions. *In order to determine with certainty from what segment the ash is derived, the correlation between the ash and the segment must be sought in one indentical section.* The position of the segments should accordingly be determined, prior to microincineration, in relation to well-defined reference points, which after the micro-incineration can be clearly observed in the spodogram. A suitable procedure is first to photograph the unstained section in ultraviolet light and then to micro-incinerate the same section. Nucleoli (or some other outstanding cellular constituent) should be selected as reference points.

To use contracted muscles in order to locate the segment which has yielded the ash is likewise a highly unsatisfactory method. During the contraction very marked changes take place in the distribution of the substances which build up the striated muscles. For example, according to CASPERSSON and THORELL (loc. cit.), the adenylic acid in the isotropic segments migrates, down the myosin fibres, to the anisotropic segments.

In order to ascertain from what segment in the striated muscles the ash is derived, *such muscles have been examined by the author with ultraviolet photography, followed by micro-incineration of the same section.* With a view to secure clear results, the muscles used in seeking this correlation were those in which the partitions were higher than in normal mammalian muscle. Suitable objects of investigation are, for example, the bone muscles from certain dipteran flies (especially *Drosophila funebris*) and the longitudinally running subcutaneous muscles of certain dipteran larvae (*Chironomus Thummi*). The striated muscles of frogs and rats have also been examined. In some cases recourse was had also to ultraviolet absorption measurements and examination in polarized light.

Technique.

Ultraviolet Microphotography and Examination in Polarized Light.

The apparatus for ultraviolet microphotography consisted of a microscope with a quartz system according to the method of KÖHLER, and the monochromatic light used had a wave-length of 2 570 Å, at which wavelength the nucleic acids reach their absorption maximum. In a few cases the wave-length 2 750 Å was also used.

The tissues, embedded in paraffin, were sectioned and mounted on plane quartz plates and cleaned in bichromate sulphuric acid. Previous to the ultraviolet photography, the paraffin was removed with chloroform and the preparation was then transferred, via absolute alcohol, into doubly distilled glycerine.

With a view to the examination of the thigh muscles of *Drosophila funebris*, the flies were anesthetized with ether or chloroform and the muscle bundles were dissected under a dissection microscope and were then photographed, in a live state, on quartz plates, at 2 570 Å. These preparations were thus not exposed to the chemical action of any fixative.

In the ultraviolet photography of the unstained preparations of striated muscles a maximally open condenser diaphragm was employed. According to CASPERSSON and THORELL (1942), the visible striation is then partly determined by a real absorption of light and not only by refraction, as in visible light. The said authors found that the striation was best marked at 2 500—2 850 Å. Below 2 400 Å and above 2 950 Å the striation was scarcely observable, which indicates that the picture produced is due to light absorption. However, in order to ascertain by what substances the light absorption is entailed, complete ultraviolet absorption spectra of the various structures must be taken (see below).

In cases where the same section was to be photographed in ultraviolet and in polarized light, the rock crystal plates could not be used, as they are doubly refracting. Instead, ultraviolet transparent, non-doubly refracting glasses from Schott, type WG 8, were employed. These glasses had the drawback that they were not well suited for micro-incineration, as they were liable to changes during the heating. The photography in polarized light of the same cellular constituent which had previously been photographed in ultraviolet light was made, in the usual way, with crossed Nicol's prisms.

Absorption Measurements.

The measurement of the ultraviolet absorption spectra was made with the apparatus and technic devised by CASPERSSON (see CASPERSSON, 1940). In regard to the analysis of the curves obtained and details of the absorption measurement, the reader is referred also to CASPERSSON 1936, 1940 and CASPERSSON and THORELL, 1942.

Micro-Incineration.

The micro-incineration was carried out in an electric furnace, the construction of which is shown by Fig. 1. The heat-insulated walls of the furnace chamber are made of well burnished, stainless steel. The chamber can be made gas-tight by frimly screwing up a shutter with an aluminium packing. As the aluminium has a higher coefficient of expansion than the screws, the chamber will be perfectly gas-tight when the temperature in the furnace has risen. In order that the combustion may proceed in different gas atmospheres, the furnace chamber is provided with two tubes. The thermo-element is inserted in the tube at the rear.

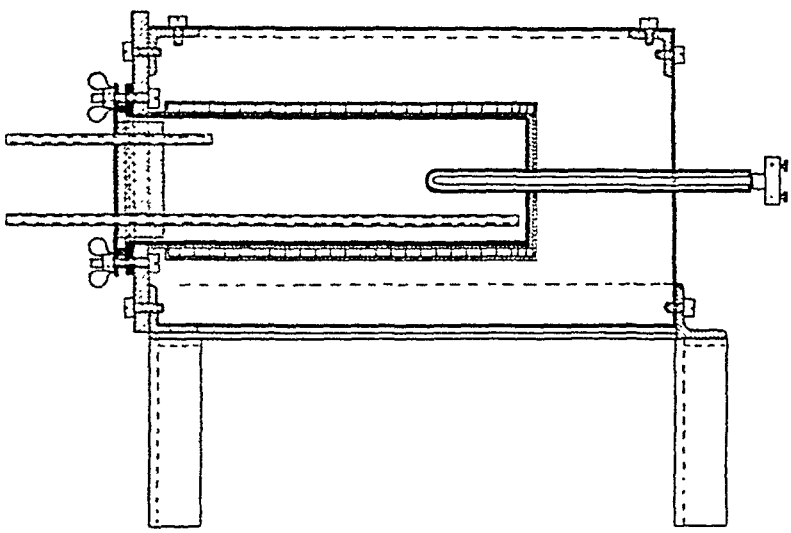


Fig. 1. Furnace for incineration. See the text.

In order to locate the structure from which the ash was derived, the same section which had previously been photographed in ultraviolet light was incinerated. For this purpose, the glycerine was completely removed with absolute alcohol. In some cases, sections were micro-incinerated without previous photography in ultraviolet light. As regards the last mentioned sections, the paraffin was removed with chloroform.

As regards the muscles from *Drosophila funebris*, the fibres were dissected without the use of any isotonic solution, and were immediately incinerated.

The incineration temperature was maintained at about 500 degrees centigrade; the duration of the combustion varied with the thickness and nature of the incinerated material. The sections were incinerated in a closed space in the presence of air.

The optical examination of the ash was made with the aid of a Zeiss cardioid condenser.

The Heat Stability of Some Compounds of Phosphoric Acid.

From the data obtained in the macrochemical analyses of striated muscles, it may be anticipated that, after the incineration of striated muscles, the anions in the ash will consist mainly of phosphates. It seems therefore desirable to discuss the heat stability of certain compounds of phosphoric acid.

In striated muscles from frogs, the cations, as compared with the anions, show a surplus of 130 milli-equivalents to 80, per kg of healthy tissue (FENN, 1936). The percentages of the principal cations and the total content of phosphorus in striated muscles from frogs are shown in the following table (FENN, 1936, DUBUISSON, 1942).

K	Na	Mg	Ca	Total P
0.32	0.06	0.02	0.02	0.15—0.20

The values in the table are in percentage of moist substance. The major part of the phosphates after the micro-incineration is probably obtained in the form of meta- and pyro-phosphates (see below). In that case the cations would presumably suffice to bind the greater part of the phosphorus, even the organic phosphoric acid liberated on incineration in the form of inorganic phosphates.

ASHTON (1936), in his study of the phosphorus content in vegetable material, contends that no phosphates are lost on heating to 600 degrees centigrade. BAGINSKI (1938) states that the orthophosphates of the alkaline metals are converted into pyrophosphates on heating to 500 degrees. NORBERG (1942), in elaborating an ultramicro method for the determination of phosphorus in biological material, thoroughly investigated the importance of the mineralization. In model experiments with KH_2PO_4 (amounts of $0.1—1.10^{-2}$ mg), NORBERG found that, after heating to 500 degrees centigrade for one hour, 62 per cent. of the phosphorus in the samples was recovered.

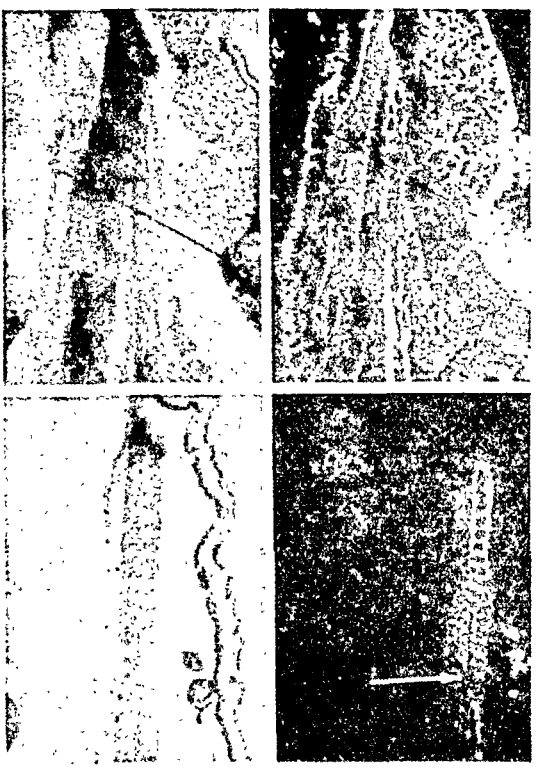
In model experiments with KH_2PO_4 (amounts of about 100 mg), the loss of weight, after heating to the same temperature as that used for the micro-incineration, was 13.6—13.8 per cent. This corresponds well to the formation of KPO_3, when a loss of weight of 13.2 % is entailed by the elimination of the water. The ortho- and pyrophosphates merely lost their water of crystallization. On the combustion of sodium thymonucleate (amounts of about 10 mg), a residue consisting, as to 25 per cent., of dry substance was obtained. If we reckon with a formation of Na PO_3, this residue would amount to about 26 per cent.

From the above it thus follows that the major part of the phosphates is retained, on micro-incineration.

Experimental Results.

Chironomus Thummi.

In order to be in a position to compare the ultraviolet and micro-incineration pictures in the same identical section and to determine with certainty from what cellular structure the ash is derived, one should use a material in which the muscle partitions



a b

Fig. 2.

a. Ultraviolet pictures of an unstained preparation of a few muscle fibres from *Chironomus Thummi*. The photographs taken at 2570 Å, with a maximally open condenser diaphragm. It will be noticed that the absorbing substance is located in certain segments. In the lower picture it can be observed that the absorbing substance is concentrated at the edge facing the weakly absorbing segments. A fine absorbing streak runs in the middle of the light segments.

b. The same sections after micro-incineration, photographed in the dark field. The upper picture clearly shows that the ash is derived from the intensely absorbing segments, whereas those segments which are not selectively absorbing are practically free from ash. The line is drawn between two well-defined points in the preparation. The two lower pictures likewise distinctly show that the ash is yielded by the intensely absorbing segments. In the ash-free segments, which correspond to the non-absorbing ones, a fine streak of ash is observed. The arrow indicates identical points in the muscle fibres. Magnification 200 × (the lower ash picture somewhat less).

are, relatively speaking, of considerable height. A material particularly well suited for this purpose was found to be the longitudinally running musculature in the larvae of *Chironomus Thummi*. In some muscle fibres the partitions are 15 μ or more in height, whereas in other fibres the height of the inocommata is considerably less.

The ultraviolet picture shows that the muscle fibres are built up of alternating dark and light segments. In the darker segments

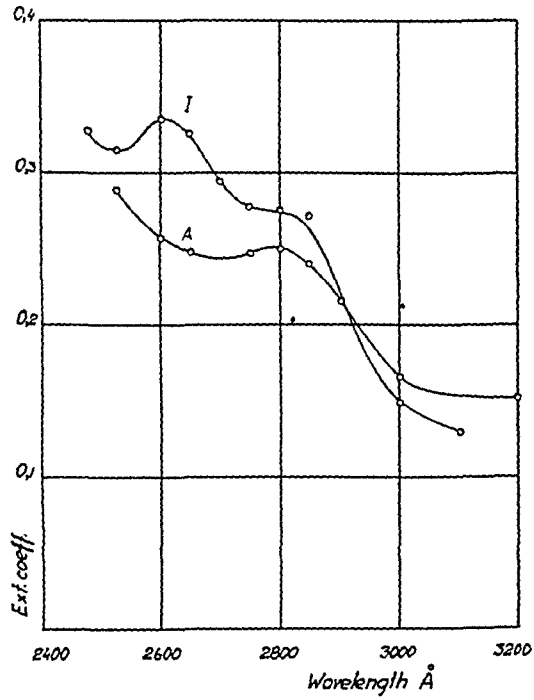


Fig. 3. Absorption spectra of an intensely absorbing (isotropic) segment, I, and a weakly absorbing (anisotropic) segment, A, adjacent to one another. *Chironomus Thummi*, thickness of section 7 μ. (Compare the text).

the absorbing substance is often concentrated at the edge facing the less intensely absorbing segments. In the middle of the non-absorbing segments, a fine absorbing streak can sometimes be observed (see Fig. 2, ultraviolet picture of unstained preparation at 2 570 Å.). In order to obtain some idea as to the nature of the absorbing substance, ultraviolet absorption spectra were taken of dark and light segments. The result of such a measurement of a dark and light segment, adjacent to one another, is shown in Fig. 3. The dark segment, in accordance with the investigations of Caspersson and Thorell, shows a distinct maximum at 2600 Å, indicating the presence of nucleotides. The light segment gives an

almost pure protein spectrum. The protein content is about the same in the two segments. The curves also show that the difference in absorption between the different segments is most marked at about 2 600 Å. Below 2 400 Å and over 3 000 Å the two curves approach one another. This signifies that the striation which is visible in an ultraviolet microphotograph taken at 2 570 Å (see the section on technique) is largely due to a real light absorption, caused by nucleotides. In this respect it contrasts with the picture of the striations obtained from an unstained preparation in visible light. The latter picture results solely from differences in refraction between the different segments.

The statement made by CASPERSSON and THORELL (loc. cit.) that it is the isotropic segments which absorb intensely could be confirmed by the author. An ultraviolet and double refraction picture from one identical section is shown in Fig. 4. The lines for the identification of the segments are drawn from well-defined points in the preparation.

a b

Fig. 4.

a. Ultraviolet picture (2570 Å) from a section of the subcutaneous muscles of a *Chironomus* larva.

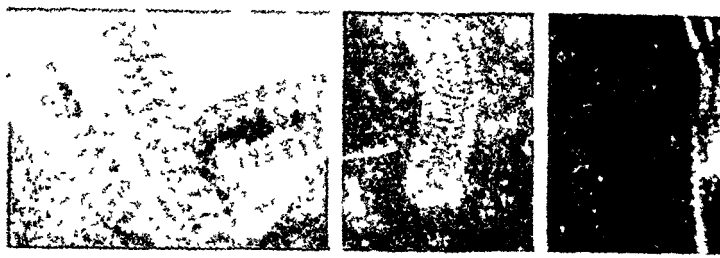
b. Double refraction picture, in visible light, of the same section. The lines are drawn from well-defined points in the preparation. The picture shows that the weakly absorbing segments are the anisotropic ones. Magnification: a. 300 ×, b. circa 200 ×.

Fig. 2 shows also the correlation between the ultraviolet absorption and the ash content in one and the same section. It strikingly shows that *the ash is derived from the ultraviolet absorbing segments.* In such segments it can sometimes be observed that the ash in certain places is concentrated at the edge facing the segments yielding less ash. The narrow absorbing streak in the middle of the light segments is seen also in the ash picture. *The light segments otherwise are practically devoid of ash.*

Summing up, it may thus be stated that in the striated muscles of *Chironomus Thummi* there is a distinct correlation between ultraviolet absorption and ash content, in that the intensely ultraviolet absorbing, isotropic segments regularly yield ash. The anistropic segments, which are not selectively absorbing at 2 600 Å, are practically devoid of ash.

Drosophila Funebris.

The leg muscles of *Drosophila funebris* afford good opportunities for experiment, as the individual muscle fibres can be free-dissected under a dissection microscope and their structure examined, without having been subjected to any fixative or solvent.



a b

Fig. 5.

a. Muscle fibres from *Drosophila* directly prepared and photographed in a living state at 2 570 Å.
b. Incinerated muscle fibres from a fruit-fly, photographed in the dark field Magnification: a. 720×, b. 500×.

Fig. 5 a. shows a few muscle fibres from the fruit-fly prepared in the above described manner and immediately photographed, in a live state, in ultraviolet light with a wavelength of 2 570 Å, whilst lying in a physiological salt solution on a quartz plate. It can be distinctly seen that the ultraviolet absorbing substance, which, as found by CASPERSSON and THORELL, consists of nucleotides, is located in certain segments. According to those authors, they correspond to the isotropic segments. The segments which come out dark on the ultraviolet photograph are separated by light ones — the anisotropic segments.

Fig. 5 b. shows a few other muscle fibres from a fruit-fly, incinerated immediately after preparation. It will be noticed that the striated structure is well preserved and that segments rich in ash alternate with those which are practically devoid of ash. As it is not possible to obtain an absoutely certain correlation between ultraviolet absorption and ash content in such detached muscle fibres, sections of fruit-flyes were also used. The flies were fixed in accordance with Gersh's freezing drying method. The results of comparison between the ultraviolet and ash pictures correspond to what has been stated above.

The experiments show that, in fixed material from *Chironomus Thummi*, there is a distinct correlation between ultraviolet absorption and ash content. In order to avoid the source of error which might arise from the possible dissolution of some substances during the fixing or subsequent treatment (that this was not the case with the adenylic acids is evident from the absorption curves), fresh, directly prepared muscle fibres (*Drosophila funebris*) which had not been subjected to any fixative or solvent were micro-incinerated. In these preparations the striated structure was distinctly visible and segments rich in ash alternated with those which were practically devoid of ash. *Seeing that certain segments in the directly prepared muscle fibres, treated without any fixative or solvent, were practically free from ash, the segments rich in ash must be of the same nature as those in fixed tissue, and thus isotropic.*

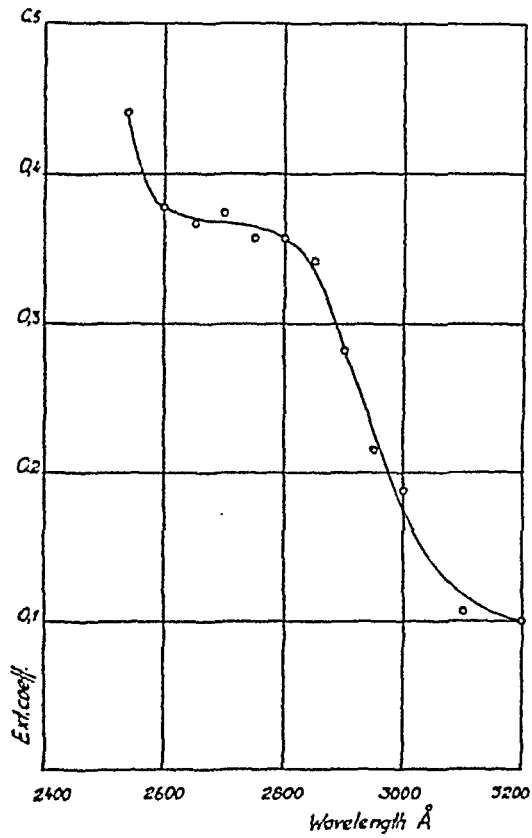


Fig. 6. Absorption spectrum of section (thickness 7 μ) from striated frog muscle. Taken with such small resolving power that the striation has no influence on the course of the curve. See the text.

Frog Muscles.

As a large number of macrochemical analyses have been made on frog muscle, striated muscles from frogs were also investigated, with a view to comparison of the results with the values obtained in the present study.

Owing to the low height of the muscle partitions, it is not possible to make an absorption measurement of an individual segment. Instead, a larger area was measured with an objective of such small resolving power that the striation had no influence. The result thus obtained is shown in Fig. 6. After correction for light-dispersion and reflection, it consists of a protein curve with

a superimposed nucleotide curve. The interpretation of the curve presents certain difficulties in regard to the calculation of the light-dispersion and reflection. In this case, evidently, we are not concerned solely with losses of light-dispersion according to Rayleigh's law: a rather considerable light reflection must also be taken into account. In order to resolve the curve obtained into a protein and a nucleic acid curve — in this case it is the nucleic acid component that is sought — it is necessary that the composition of the protein should be known. The protein band at 2 800 Å is determined especially by the content of certain aminoacids, namely tyrosin, phenyl-alanin and tryptophan (see CASPERSSON 1940). As shown by BAILEY (1937), the percentage of these aminoacids is surprisingly constant in myosin from different animals and animal groups. The tyrosin contents amounts to 3.5—4 % and the tryptophan content to about 1 %. Taking into account the factors just mentioned, we obtain a value of the magnitude of $0.1 \cdot 10^{-11}$ per μ^3 for the amount of adenylic acids, which corresponds rather well with the values previously obtained by macrochemical analysis.

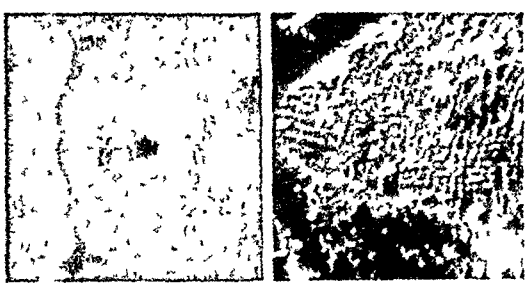


Fig. 7. a and b. Ultraviolet picture (2570 Å) and micro-incineration picture (in the dark field) of a section from striated frog muscle. Magnification circa 700 ×.

In ultraviolet, the striation of skeletal frog muscles is brought into clear relief (see Fig. 7). According to the investigations made by CASPERSSON and THORELL on live muscles of frogs, the ultraviolet absorbing segments are the isotropic ones. In the anisotropic, less intensely absorbing segments, a little, narrow absorbing streak can sometimes be observed. The micro-incineration picture (Fig. 7 b, not the same section as Fig. 7 a) illustrates the alternation of segments rich in ash with those which are practically devoid of ash. A fine streak of ash can sometimes be seen in the otherwise ash-free segment.

Survey and Discussion.

In previous micro-incinerations of striated muscles, several authors have found that the striated structure was well preserved

in the ash. In regard to the localization of the ash, no exact investigations had apparently been made. On the basis of comparison between a microincinerated and an adjacent stained section, several authors (TSCHOPP, 1929, SCOTT 1932/33, LORETI, 1940 etc.) contended that the ash was located in the anisotropic segments. This statement, however, is contradicted by the results which CASPERSSON and THORELL obtained with more exact methods, namely that the adenylic acids are located in the isotropic segments. These acids in fact occur in such large amounts that, on micro-incineration, the ash which they yield should be distinctly visible, and thus situated in the isotropic segments.

In order to ascertain the precise structure which yields the ash, the same indentical section was investigated with ultraviolet microphotography and micro-incineration. The segments which came out dark in ultraviolet light with a wave-length of 2570 Å were also subjected to absorption measurements, whereupon, in accordance with the findings of CASPERSSON and THORELL, it was ascertained that the absorbing substance was of the nucleotide type. According to those authors, the ultraviolet absorbing substance is situated in the isotropic segments; this finding was likewise confirmed.

Comparison between the ultraviolet and the micro-incineration pictures for one and the same muscle section (*Chironomus Thummi*, high muscle partitions) showed that the ash was derived from the intensely absorbing segments, whereas the weakly absorbing segments were practically devoid of ash. On muscle fibres dissected from fruit-flies and examined without their having come into touch with any fixative or solvent, it was ascertained that segments rich in ash alternated with those which were practically free from ash. In view of this observation, it is evident that the location of the ash from these dissected muscle fibres must be the same as that of the ash from sections: *the segments rich in ash must thus be the isotropic ones.*

Out of the total amount of phosphates contained in the ash from resting striated muscles, it may be estimated that 20—30 % comes from the adenylic acids, which are situated in the isotropic segments. The major part of the phosphates in the ash, 50 % or more, is estimated to be derived from the phosphagene. In regard to the analysis of the phosphoriferous constituents in striated muscles from different animals and animal groups, see MEYERHOF, 1930, PALLADIN, 1931, RIESSER and HANSEN, 1933, EGGLE-

TON, 1933, BALDWIN, 1933, FENN, 1936, etc. *Seeing that the non-absorbing segments were practically devoid of ash, it seems probable that the major part of the phosphagene is situated in the ultraviolet absorbing segments.* The possibility that the phosphagene is located in the same isotropic segments as the adenylic acids is also indicated by the close interplay between these two substances (see EGGLETON, 1935, LUNDSGAARD, 1938 and MILLIKAN, 1942).

Summary.

Comparative ultraviolet and micro-incineration studies of striated muscles have been made by the author. In order to obtain a distinct correlation between ultraviolet absorption and ash content, a material was used in which the muscle partitions are of considerable height (the leg muscles from *Drosophila funebris* and the subcutaneous muscles from larvae of *Chironomus Thummi*). One and the same section was used for ultraviolet microphotography (wave-length 2570 Å) and microincineration, which made it possible to obtain an exact localization of the ash. The result of the comparison was the finding that ash was regularly yielded by the intensely absorbing isotropic segments, which, on absorption measurement, were found to contain nucleotides (adenylic acids), whereas the anisotropic, weakly absorbing segments were practically devoid of ash. On account of these experimental findings it seems probable that the major part of the phosphagene is situated in the isotropic segments.

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On the Rôle of the Thyroid in the Methylation of Glycocyamine.

By

POUL ASTRUP and GUNNAR STEENSHOLT.

Received 10 May 1944.

The study of methylation processes in the animal organism has in recent years attracted an increasing number of investigators. From the large material that has accumulated in various branches of biochemistry, it appears that methylation processes are of vital importance for the life of the organism. However, their study offers great difficulties, which arise partly from our ignorance about the mechanism of the methylation processes, and also partly from technical difficulties in the determination of reaction products, etc.

Most present-day authors seem to favour the assumption that the methylation processes are enzymic in nature, the methyl groups being transferred from some donator to the compound in question by a chain of reactions, all or some of which are catalysed by some more or less specific enzymes. However, another possibility has been discussed in the literature (STUBER, RUSSMAN and PROEBSTING 1923), namely that methyl groups are transferred by means of iodine, in close analogy to the wellknown reaction so extensively used in synthetic organic chemistry. Their work was carried out with glycocyamine, since for this substance the amount of methylated product, namely creatine, can be conveniently measured. The results were highly interesting: they found that the methylation of glycocyamine in the organism of the rabbit depends in some way on the function of the thyroid. In normal animals the process takes place, but if the gland is removed the methylation is completely inhibited. If, however, thyroid extracts or iodine are injected, the organism regains its property of methylating

glycocyamine. It is clear that these results, if confirmed, would determine the direction of much of the further work on methylation problems, and at the same time give an important insight into the mechanism of the biological function of the thyroid. It should be mentioned that doubts have already been expressed about the validity of these results (GUGGENHEIM 1940), but a search of the literature available to us revealed that no experimental verification exists of the work of STUBER, RUSSMAN and PROEBSTING. We have therefore found it decidedly worth while to undertake a renewed investigation of this problem, and the present paper is a report on our results.

Experimental Part.

Methods of analysis. For the determination of the creatinine and the creatine plus creatinine content of blood we have applied the method described by LIEB and ZACHERL (1934), which runs as follows:

Reagents.

1. Cold, saturated solution of picric acid.
2. 10 % sodium hydroxide solution.
3. 10 % sodium tungstate solution.
4. 2/3 n-sulphuric acid.
5. 1 n-hydrochloric acid.

1 ml blood (made incoagulable by oxalate, citrate or heparine) is hemolysed in 2 ml of water. 1 ml 10 % sodium tungstate solution and 1 ml 2/3 n-sulphuric acid are added and the mixture is thoroughly shaken. After about 20 minutes the precipitate is spun down in the centrifuge.

a) *Creatinine.* 2 ml of the protein-free filtrate are added to 4 ml picric acid solution in a 10 ml flask. 0.4 ml 10 % sodium hydroxide is added, and the mixture allowed to stand for 10 minutes. Distilled water is added until the total volume is 10 ml, and the colorimetric measurements are carried out immediately afterwards in the ususal manner in a Pulfrich photometer.

b) *Total creatinine.* 2 ml of the protein-free blood filtrate are filled into a 10 ml flask. After addition of 0.4 ml n-hydrochloric acid the mixture is heated in an autoclave at 130° for 20 minutes. After cooling the liquid is filtered if not clear, and 4 ml picric acid and 0.4 ml 10 % sodium hydroxide solution are added. After standing for 10 minutes water is added up to the 10 ml mark, and the colorimetric measurements carried out as above.

The method of LIEB and ZACHERL has also been modified by these authors to apply to the determination of creatine and creatinine in urine. For details of the procedure the reader is referred to the original paper.

Substrates. The glycocyamine used in this work was synthesised by the method of NENCKI and SIEBER (1878), which was found to work very smoothly and with satisfactory yield.

Experimental animals. All our experiments were made on rabbits, as was also done by STUBER, RUSSMAN and PROEBSTING. The extirpation of the thyroid was carried out under ether narcosis. This operation presents the well-known difficulties of securing a complete extirpation of all glandular tissue. We have, however, regarded the symptoms of the animals after they recovered from the operation (particularly the appearance of their furs, which became very shabby, with yellowish spots) as convincing proof of the success of the operations.

Experimental procedure. All experiments were carried out in the following manner: To begin with, a blood sample was taken from an ear vein, with heparine as anticoagulant. Then an intravenous injection, also in the ear, was given of 100 mg glycocyamine dissolved in about 15 ml 0.9 % NaCl solution. The injections were completed in about $^1/_2$ minute. Thereafter blood samples were taken as described above at suitable intervals. All the analyses were carried out immediately after the experiment had been completed. Double analyses were carried out in all cases; the results always agreed within 5 %.

Results. To begin with a blind experiment was carried out to see whether the injection of the NaCl-solution had any influence on the colorimetric measurement of creatine and creatinine. This was found not to be the case. In the same way we found that the addition of glycocyamine to a blood sample in vitro had no effect on the creatine-creatinine determinations, at any rate not in the concentrations used in our experiments.

We first investigated the variations in preformed blood creatinine after injection of glycocyamine in altogether 18 normal animals. After the injections at least three and usually four or five blood samples were taken. A typical experiment, chosen at random, is the following:

Table 1.

Experiment no. 14. Male. Weight: 2.7 kg.

	Preformed blood creatinine in mg %		
	1. analysis	2. analysis	Average
Before the injection	3.50	3.63	3.57
5 min. after the injection	4.12	3.93	4.03
14	3.65	3.59	3.62
35	3.02	3.17	3.10

In this experiment the difference between the highest preformed creatinine value after the injection and the value before the injection amounts to 13 per cent of the latter. In all the animals in-

vestigated this quantity ranges from — 9 to + 13 per cent, and clearly without any regularity at all. As a rule the highest value was found in the first blood sample taken after the injection, but we did not observe any correlation with sex or with body weight of the animals. Thus, no definite conclusions could be drawn from these experiments. We therefore decided to determine the total blood creatinine as well, and in this way a complete investigation of 7 normal animals was carried out. An example is given in Table 2, and the others are qualitatively of exactly the same type. Similar experiments were carried out on 7 thyreodectomised animals. Table 3 contains a typical example. 5 others were exactly similar, and only one of the thyreodectomised rabbits failed to respond to glycocyamine injections.

We also analysed the urine of these animals for creatine-creatinine, but the results were so variable and inconsistent, that they did not allow us to draw any definite conclusions, and they are therefore not reproduced here. The reasons for this are not quite clear at the present moment, and must be left for further discussion at another occasion.

Table 2.

April 3., 1944. Normal Male. Weight: 2.7 kg.

	Preformed creatinine mg%	Total creatinine mg%	Creatine mg%
Before injection	3.89	5.85	1.96
10 min. after injection	4.03	7.63	3.60
20	4.15	6.86	2.71
65	3.58	5.63	2.05
120	3.65	5.88	2.23

Table 3.

March 29., 1944. Female. Weight: 2.77 kg. Thyreodectomised March 11., 1944.

	Preformed creatinine mg%	Total creatinine mg%	Creatine mg%
Before injection	4.16	4.97	0.81
6 min. after injection	4.22	7.74	3.52
16	4.18	7.10	2.92
47	4.13	7.00	2.87
120	4.11	6.04	1.93

Discussion.

As already mentioned the results for preformed creatinine were so variable that they are unsuitable as a basis for further discussion. It must be said, however, that even this very circumstance is in contradiction to the results of STUBER, RUSSMAN and PROEBSTING. They always found a rise in preformed creatinine in normal animals, and, moreover, the preformed creatinine returned exactly to its value before the glycocyamine injection in about 1 hour. Both these features are lacking in our experiments. This disagreement does not seem to be explicable by differences in the methods; the principle underlying our method is the same as that of the procedure used by STUBER, RUSSMAN and PROEBSTING, only the technical details being different. Furthermore, it seems to us worth pointing out explicitly that the regularity in the variations of blood creatinine found by the latter authors is of a perfectness and an almost ideal precision that is hardly to be expected in biological work. Finally we note that we did not find any difference in the variations of the preformed blood creatinine in normal and thyreodectomised rabbits after injection of glycocyamine. This is also quite contrary to the findings of STUBER, RUSSMAN and PROEBSTING.

Turning now to the results for creatine, a glance at the tables shows that both normal and thyreodectomised animals behaved in exactly the same manner: an injection of glycocyamine is followed by a rapid and very pronounced rise in creatine. Only one out of seven thyreodectomised rabbits failed to respond in this way to an injection of glycocyamine, but this single failure can hardly affect our main conclusion. It can therefore be stated that also this part of our results is in direct contradiction to the results of STUBER, RUSSMAN and PROEBSTING. In all cases of thyreodectomised rabbits the latter authors found absolutely no trace whatever of a methylation of glycocyamine.

We are thus unable to confirm the results of STUBER, RUSSMAN and PROEBSTING concerning the rôle of the thyroid in methylation processes; at all essential points the two sets of results are irreconcilable.

Summary.

The paper is a report on a reinvestigation of the rôle which the thyroid, according to some workers, is supposed to play in the methylation of glycocyamine. The authors found that the gland has no effect whatever on this process, normal and thyreodectomised rabbits reacting in the same way to glycocyamine injections, and were therefore led to reject the results of STUBER, RUSSMAN and PROEBSTING together with their theory of methylation processes.

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From the Physiological Departement of Physiology,
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The Activity of the Gastric Secretory Excitant of the Pyloric Mucosa after Treatment with Histaminase.

By

ÅKE BAUER and BÖRJE UVNÄS.

Received 11 May 1944.

Strong experimental evidence has accumulated in favour of the view that the gastric phase of gastric secretion is regulated by a hormonal mechanism. According to EDKINS' "gastrin theory" the gastric hormone is released from the pyloric mucosa. Many workers have reported the existence of a gastric secretory excitant in acid extracts from the mucosa of this region. In 1920 POPIELSKI showed that histamine stimulates the gastric glands. As the active extracts prepared by previous workers exerted vasodilator properties this was taken to indicate that the active principle of the extracts of EDKINS and following authors had been histamine. In 1932 SACHS, IVY, BURGESS and VANDOLAH succeeded in isolating crystalline histamine from acid extracts of the pyloric mucosa of pigs. The extracts lost their excitatory properties when incubated with histaminase. According to SACHS and collaborators "our experiments offer strong evidence in favour of the view that in acid extracts of the pyloric mucosa histamine is the sole gastric excitant which is active when the extracts are introduced subcutaneously". These observations were taken to indicate that histamine was identical with the gastric hormone. Since then this view has been widely accepted. KOMAROV (1938, 1941) and UVNÄS (1942, 1943) and MUNCH-PETERSEN, RÖNNOW and UVNÄS (1944), however, have isolated from the pyloric mucosa a gastric secretory excitant not identical with histamine. Experimental facts strongly suggest that the secretory agent extracted by these workers is identical with the gastric hormone "gastrin". Considering the

report of SACHS and collaborators it seemed of interest to investigate the resistence of our pyloric secretory excitant to histaminase.

Experimental.

The secretory agent was obtained from HCl-extracts of the pyloric mucosa of cats and pigs. A crude active material was obtained from cats by simple precipitation with trichloracetic acid (UVNÄS 1943). The material from pigs was purified according to the method of MUNCH-PETERSEN, RÖNNOW and UVNÄS (1944). A histaminase of reliable activity prepared by SVEDIN (1943) was used.

The pyloric preparations were incubated with histaminase in the following way. An amount of dry material sufficient to evoke a copious gastric secretion was dissolved in 10 ml physiological saline. The pH of the solution was brought to 8 by adding 5 ml of a Sörensen phosphate buffer or a few drops of N/10 NaOH, 0.5 ml of a solution of histaminase was added and the mixture allowed to stand for 15—20 hours at 37° C. The secretory activity was then tested by slow intravenous injection on narcotized cats as devised by UVNÄS (1943). In control samples the histaminase was omitted and 0.5 ml of physiological saline substituted. In special experiments the activity of the histaminase was controlled. An amount of histamine, the secretory power of which corresponds to the pyloric preparations, was incubated with histaminase as devised above. The remaining histamine activity was then estimated on the cat's blood pressure.

Results.

A copious gastric secretion was evoked by all of the ten pyloric preparations incubated with histaminase. As seen in the experiment illustrated in fig. 1, incubation for 15 hours of a pyloric preparation from cats with histaminase did not reduce the activity of the secretory principle. During the same time under identical conditions 500 γ histamine was completely and 1000 γ almost completely broken down, only 50 γ being left.

The estimations of the secretory activity of the preparations was interfered with by the fact that in several experiments the histaminase showed inhibitory properties. After testing a material containing histaminase the response to pyloric preparations or to histamine was considerably decreased in the majority of the experiments. The inhibition did not set in immediately. The preparations incubated with histaminase evoked a copious gastric secretion. Several tests on one and the same animal could not

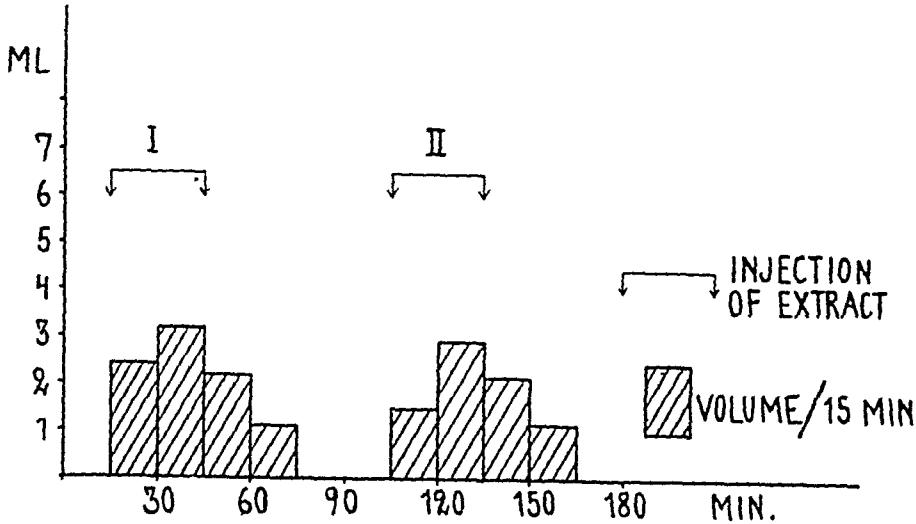

Fig. 1. Gastric secretion after slow intravenous injection of 100 mg of a pyloric preparation from cats.
I. Control, not incubated with histamine.
II. Incubated with histaminase for 15 hours.

however be performed. The inhibitory effect which was not abolished by inactivation of the histaminase was probably due to some impurities.

Discussion.

In our experiments the pyloric gastric secretory excitant is resistant to histaminase. Quantities of histaminase which do not affect the secretory power of a pyloric preparation destroy an amount of histamine of corresponding secretory activity. This seems to be contradictory to the experiences of SACHS and collaborators. They found that acid extracts of pyloric mucosa were inactivated when incubated with histaminase. In a previous paper UVNÄS (1943) made an observation which may explain the discrepency between our results and those of SACHS and collaborators. It may also explain the fact that the pyloric principle has remained undiscovered for so long. UVNÄS found that gastric secretion was evoked only if the pyloric principle was administered intravenously. Subcutaneous injection was quite ineffective. Many previous workers a. o. SACHS and collaborators injected their mucosal extracts subcutaneously. Apparently they only observed the secretory effect of histamine which effect naturally disappeared after incubation of their extracts with histaminase.

Summary.

The gastric secretory agent described in previous papers from this laboratory is resistant to histaminase.

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Insulin and the Assimilation of Fructose.

By

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The question of whether insulin exerts any influence on the assimilation of fructose as it does on glucose assimilation is answered rather variously by different authors. Nevertheless the majority of investigations in this field seem to indicate that insulin has no effect on fructose assimilation.

One of the means chosen to attack this problem is the determination of the fructose tolerance before and after the administration of insulin. BASCHE and POLLAK (1922) performed experiments of this kind on rabbits. The fructose was injected in varying doses intravenously in the course of a minute. The urine was collected and the power of reduction determined. By this method the authors found a moderate but distinct increase of the fructose tolerance after insulin. CORI and CORI (1927 b) made corresponding investigations on rats, though here the method employed was a continuous intravenous infusion of fructose. Whereas similar experiments with glucose revealed a marked increase of the glucose tolerance after insulin, these authors were unable to observe a corresponding effect on the fructose tolerance. In an earlier work CORI (1927) had demonstrated that large doses of insulin reduce the deposition of glycogen in the liver after the oral administration of fructose. The corresponding effect of insulin on the glycogen deposition in the liver after the oral administration of glucose had already been demonstrated in a somewhat earlier work by CORI and CORI (1927 a). In this latter work the authors write as an explanation of the reduced deposition of glycogen in the

liver after insulin: "The muscles appropriate so much sugar that there is nothing left to be stored in the liver." If this explanation is to hold good as regards the inhibitory effect of insulin on the deposition of fructose in the liver, the insulin would not merely promote the assimilation of glucose in the muscles but that of fructose as well. This does not agree with CORI and CORI's later experiments, in which it was not possible to demonstrate any effect of insulin on the fructose tolerance. The insulin doses were of the same very high order in both series of experiments.

In experiments on rabbits CORLEY (1929) determined the rate at which fructose is eliminated from the blood after a single intravenous injection of this sugar. The author concludes that the assimilation to some extent is increased in animals who receive insulin for some time prior to the fructose injection. It is doubtful, however, if the results of his experiments bear out this conclusion. Similar experiments were made by DAVIDSON et al. (1936) in which no effect of insulin was observed on the rate of disappearance of fructose from the blood. The same result was arrived at by WIERTZUCHOWSKI et al. (1931) in experiments on dogs. These authors employed continuous intravenous infusion of fructose over a period of three hours. On some days the experiments were made without insulin, and on other days experiments were made on the same animals after insulin. The average curves of the fructosaemia in the control experiments and the insulin experiments differ only very slightly. The observation made by HERBERT and DAVIDSON (1938) that the course of fructoseaemia in diabetics after oral administration of fructose does not differ from the course in normals may also be mentioned as one of the observations that indicate that insulin has no effect on the assimilation of fructose.

In in-vitro experiments with the diaphragm of rats GEMMILL (1941) showed that insulin promotes the storing of glucose in the form of glycogen in the muscular tissue. In corresponding experiments with fructose as the medium there was found to be no glycogen deposition, with or without insulin. As it may be taken for granted that fructose can be stored as glycogen in muscular tissue, the absence of any insulin effect in GEMMILL's experiments is perhaps of small value in support of the view that insulin exerts no effect on the assimilation of fructose.

In the afore-mentioned work by DAVIDSON et al. (1936) the authors, after recounting the observations that suggest that in-

sulin has no effect on fructose assimilation, write: "All these results are consistent with the hypothesis that the first stage in the fructose metabolism is the conversion of fructose into glucose (or some similar substance) and that the rate of this conversion is independent of insulin, while the second stage is the disposal of the glucose (or similar substance) and is controlled by insulin."

The validity of this simple explanation or formulation is open to doubt, however. At any rate it is doubtful if the fructose is converted into free glucose prior to its conversion into glycogen. A weighty argument against it is the observation of LUNDSGAARD et al. (1936) that glucose is not stored in the artificially perfused isolated cat liver, whereas fructose is readily stored as glycogen. When fructose is added to the blood perfused on isolated cat liver the power of reduction falls rapidly and the glycogen content of the liver rises. This fall in the blood's power of reduction however caeses before it has got down to the same level as prior to the addition of the fructose. At the end of the experiment it is demonstrable that the blood's power of reduction is due solely to glucose. Part of the added fructose (approximately half) is deposited in the liver as glycogen, whereas the remainder is found as glucose in the blood. On repeating these experiments in conjunction with the experiments reported on in the present paper the earlier result was confirmed. In these new experiments the fructose concentration in the blood was traced throughout, whereby it was possible to show that the fall in the blood's power of reduction — and consequently the deposition of glycogen — ceases at the moment the last trace of fructose in the blood disappears.

Although fructose is assimilated in the muscles, there is nothing to show that it is converted into glucose in the muscular tissue. This was already pointed out by S. ISAAC in 1914 (unfortunately his work, J. Phys. Chem. 1914, *89*, 78, is not available to us). In the following experiments with hind-leg preparations we have seen no increase of the glucose content in the blood after adding fructose, even in cases where the blood-glucose was extraordinarily low. It is extremely probable that the fructose passses from the blood into the muscle cells as fructose.

Several authors, including LUNDSGAARD (1939), favour the assumption that the primary effect of the insulin consists in an acceleration of the passage of the glucose from the blood into the muscle cells, a passage which cannot be assumed to be a simple

diffusion of free glucose. Consequently, it would be of no slight importance to our comprehension of the action of the insulin if it could be established beyond doubt that while insulin promotes the passage of glucose into the muscle cells, it has no effect on the corresponding passage of the closely-related hexose, fructose.

We have therefore considered it worth while once more to take up the question of the effect of insulin on fructose assimilation for renewed experimental testing. For this purpose we elected to experiment on artificially perfused hind-leg preparations, for the following reasons. With such a muscle preparation the effect which we regard as the primary insulin effect can be demonstrated clearly and with great regularity. Disturbing influences from accompanying reactions in other organs are precluded. In such a preparation the concentration of the sugars tested can be set at any selected level and with great certainty can be maintained at these levels for a considerable time. Finally, the wide experience of the Institute with regard to glucose assimilation in hindleg preparations made it natural to choose this experimental object for the projected investigations into fructose assimilation.

Technique.

The experiments were made on cats which had been starved 24 hours. The artificial perfusion of the isolated hind-leg preparations was performed by the usual method of this institute (LUNDSGAARD et al. 1936). All the animals were of about the same size (weight about 3 kg).

For those experiments in which it was desired to operate with a very low blood-glucose concentration the glucose was removed by fermentation. Blood corpuscles and plasma were separated by centrifuging. About 10 g of washed yeast per 100 ml was suspended in the plasma, whereafter it stood for an hour at 38° in the incubator. The corpuscles were washed once by suspending them in 0.9 per cent. sodium chloride and renewed centrifuging. The yeast was removed from the plasma by centrifuging, and the washed corpuscles were suspended in the glucose-free plasma. As the intercellular fluid in the hind-leg preparation contains glucose, which when perfusion begins distributes itself over intercellular fluid and blood, it is impossible to obtain a blood-glucose concentration of 0. The total reduction at the beginning of the experiments was approximately 30—40 mg% glucose.

Fructose, and when desired glucose, were added to the blood at the beginning of the experiment in such quantities that the selected concentration in the blood was reached. We endeavoured to keep the concentration of the two sugars constant throughout the experiment

by a constant infusion of glucose (5—7 mg per minute) and fructose (3—10 mg per minute) from a motor-driven syringe.

In all the experiments the insulin dose was 0.5 mg of a crystalline preparation (22 units per mg).

For determining the glucose and fructose content in the blood we employed the total reduction (HAGEDORN-JENSEN's method) and total colour development with diphenylamin by the method described below.

With the HAGEDORN-JENSEN method we determined the power of reduction of fructose to be 98 per cent. of that of glucose.

With the diphenylamin method as we employed it the colour intensity developed with various glucose concentrations was 7.5 per cent of the colour intensity of the equimolar fructose concentrations.

These values allow us from the total reduction (indicated in mg% "glucose") and the colour intensity (indicated in mg% "fructose") to calculate the concentrations of glucose and fructose simultaneously present in the blood according to the following equations:

$$X + 0.98\ y = \text{the total reduction}$$
$$0.075\ X + y = \text{the colour intensity}$$

where X indicates the glucose concentration in mg% and y the fructose concentration in mg%.

For the colour development with diphenylamin and the subsequent determination of the colour intensity we followed — with slight deviations — the method described by ALVING, RUBIN and MILLER (1939) (for insulin determination).

The protein in the defibrinated blood was precipitated with $ZnSO_4$-NaOH after SOMOGYI (1930). The sediment was centrifuged off. The colour reaction was obtained with 2.5 ml of the above fluid + 5 ml reagent (consisting of 110 ml 96 % ethylalcohol + 80 ml concentrated hydrochloric acid + 10 ml 10 % diphenylamin in 96 % ethylalcohol) in a test-tube which was sealed by heat. The tubes stood exactly 60 minutes in a boiling water bath in which the water level was well above the level of the fluid in the tubes. This was followed by rapid cooling down to room temperature in cold water. Then colorimetry. In some of our first experiments we used a Bürker colorimeter, but later we employed HAVEMANN's photo-electric colorimeter, with the ordinary bulb filter RG_1 and a layer thickness of 5 mm. With this the concentration in the Somogyi filtrate was 0—12 mg%, best 2—6 mg%, which was obtained either by diluting the blood or by taking smaller quantities of blood for the protein precipitation.

Results.

The method adopted in the first experiments was to set the concentrations of glucose and fructose in the blood as far as possible at the same level at the outset. In the various experiments this level varied from 150 to 300 mg%, and we endeavoured to

maintain the level by means of a continuous infusion of glucose and fructose. Insulin was added to the blood one hour after the beginning of the experimental period. Fig. 1 A is a reproduction of the results of one of these experiments. It will be seen that the insulin has a distinct effect on the glucose assimilation, as the concentration of this sugar, which was almost constant before the insulin was added, then decreased despite the unchanged continuous addition of glucose. On the other hand, the concentration of fructose in the blood is not affected. In this particular experiment indeed the fructose concentration seems rather to rise a little more after the addition of the insulin than before. In all six experiments of this kind were carried out, and the results all agreed.

As we cannot quite rule out the possibility that insulin with predilection acts upon the assimilation of glucose, so that if it has any effect on the fructose assimilation this may escape observation when there is ample glucose in the blood, some experiments were performed in which the glucose as far as possible was removed from the blood by fermentation as described under "Technique". In these experiments we omitted the continuous supply of glucose to the blood. The concentration of fructose varied from 150 to 400 mg% in the various experiments. The continuous supply of fructose varied between 3 and 10 mg per minute, all according to the fructose concentration in the blood we were working with. When the addition was 10 mg of fructose per minute, however, the concentration, even when it lay at 400 mg%, always rose steadily throughout the experimental period. The results of an experiment of this kind are reproduced in Fig. 1 B. It appears directly from the curves that even under these conditions insulin has no influence on the assimilation of fructose.

In order to make sure that treating the plasma with yeast does not entail such changes that the insulin effect is inhibited we made two control experiments, in which glucose was added to the blood after fermentation. Here 5 mg glucose per minute was added continuously to the blood. Fig. 1 C shows the result of one of these experiments. It will be seen that the pretreatment of the blood with yeast does not prevent the typical insulin effect on the glucose assimilation. It would thus seem that insulin has no effect on the fructose assimilation in the isolated hind-leg preparation.

In addition to these experiments with hind-leg preparations

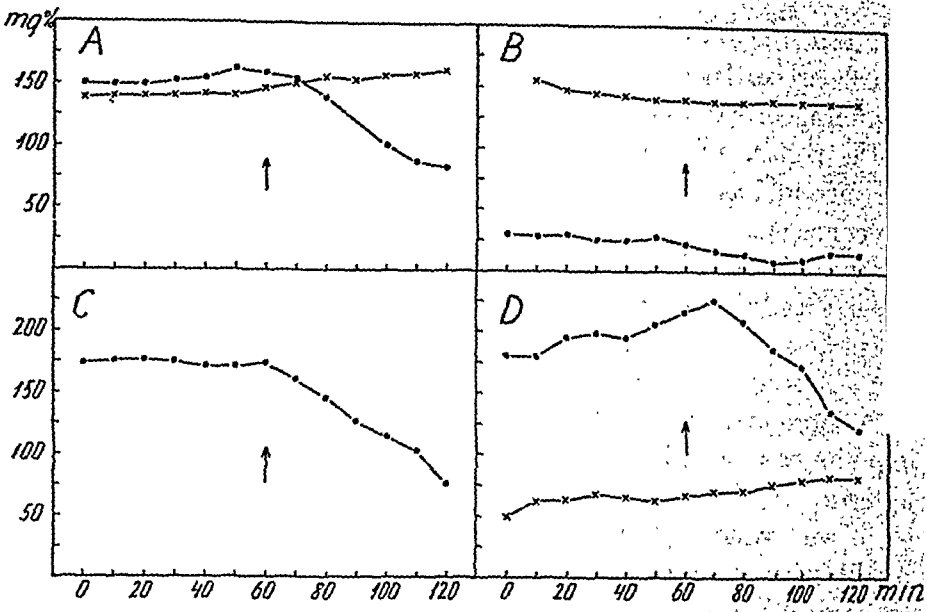

Fig. 1. The effect of insulin on the glucose and fructose assimilation in hind-leg preparations (A, B and C) and in intact animals (D). The arrow indicates the addition of insulin. Ordinate: glucose and fructose in mg%. Abscissa: time in minutes. •—•—•—• Glucose ×—×—×—× Fructose.

A. Continuous infusion of 5 mg glucose and 3 mg fructose per minute.
B. Continuous infusion of 3 mg fructose per minute.
C. Continuous infusion of 5 mg glucose per minute.
D. Continuous infusion of 50 mg fructose per minute.

we carried out some few experiments with intact animals in amytal narcosis. They were performed on the same principle as those with hind-leg preparations.

At the outset a suitable quantity of fructose was injected intravenously in order to bring the fructose concentration in the blood up quickly, whereupon we proceeded with continuous intravenous supply of fructose (50 mg per minute). Sampling began half an hour after the beginning of continuous infusion. At this juncture the fructose concentration in the blood had generally adjusted itself at a fairly constant level. The quantity of fructose infused per minute in these experiments is very high compared with those on hind-leg preparations, because the liver plays a rather dominant role in fructose metabolism. In our experiments with isolated livers we have found that with a moderate fructose concentration in the blood a cat liver metabolizes 25—30 mg fructose per minute. One hour after sampling began we injected 1 mg crystalline insulin intravenously. The results of one of these experiments are reproduced in fig. 1 D. It will be seen that

the insulin exerts the ordinary influence on the glucose content in the blood, but has no effect on the fructose content. Thus it was not possible even in these experiments with intact animals to demonstrate any effect of insulin on fructose assimilation.

The striking difference observed between the assimilation of glucose and that of fructose makes it presumable that there is a fundamental difference in the assimilation of the two sugars. For the purpose of examining to what extent the assimilation of the two is independent we made some few experiments with hind-leg

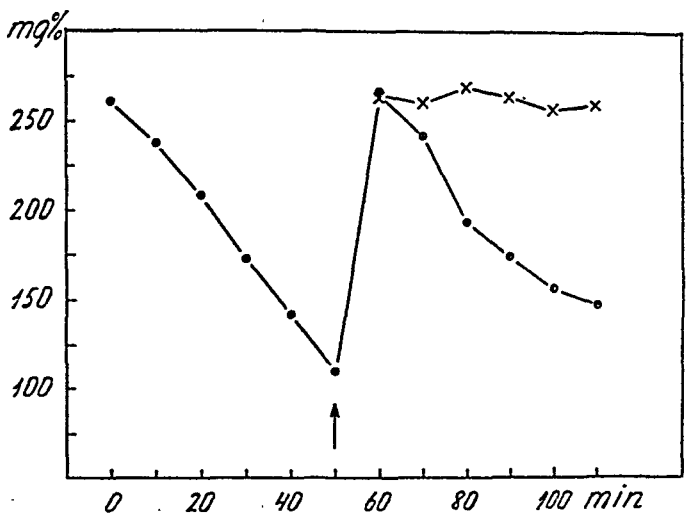

Fig. 2. Glucose assimilation in hind-leg preparation treated with insulin before and after adding fructose. At the arrow 0.75 g glucose and 1 g fructose was added, whereafter 7.5 mg fructose per minute was added continuously.

preparations in the following manner. At the outset a suitable quantity of glucose was added to the blood so as to have a fairly high initial concentration. At the same time insulin was added to the blood in the usual quantity in order to cause rapid glucose assimilation. After one hour, during which time the glucose concentration in the blood falls heavily, more glucose was added to the blood, thus bringing the concentration back to approximately the initial value. At the same time fructose was added to the blood in such a quantity that a fairly high fructose concentration was arrived at, and we endeavoured to maintain this by continuous infusion of fructose. It will be seen that the conditions for glucose assimilation in the first and second halves of the experiment were the same, except that in the second half an assimilation of fructose proceeded side by side with one of glucose. The results

of one of these experiments are reproduced in the Graphs Fig. 2 According to the procedure described by LUNDSGAARD et al. (1939 the absolute quantities of glucose assimilated per unit time ca be calculated. In the initial period 11 mg glucose per minut was assimilated when the blood sugar averaged about 200 mg% During the first 30 minutes of the second half of the experimen there was likewise an assimilation of 11 mg glucose per minut with a corresponding blood-glucose concentration, despite the fac that in this same second half 7.5 mg of fructose per minute wa assimilated at the same time. Accordingly the assimilation of glu cose and of fructose would seem to be quite independent of eac other.

Finally it may be mentioned briefly that in some of the experiments where insulin was added we determined the inorganic phosphate content of the plasma. In these experiments it was found that the well-known effect of insulin on the plasma content of inorganic phosphate, which as shown by LUNDSGAARD (1938) is also demonstrable on hind-leg preparations, stands out clearly even when the glucose content in the blood is extraordinarily low. In these experiments the glycogen desposition caused by the insulin can only have been very small at the most, for which reason the observation contradicts the view advocated by CORI (1940) that the fall in inorganic phosphate is secondary in relation to the storage of glycogen.

Discussion.

In our opinion the pronounced mutual independence of the assimilation of glucose and fructose may be explained most simply by saying that it is the rate of transfer of the two sugars from the blood into the interior of the muscle cells that constitutes the limiting factor for their assimilation. This view also harmonizes with the marked dependence of glucose assimilation on the concentration of glucose in the blood. Although in the experiments on which the present work is based we made no systematic investigations into the dependence of fructose assimilation on the fructose concentration in the blood, we consider that on the basis of our observations it is possible to state that fructose assimilation too is to a marked extent dependent on the concentration of fructose in the blood. When no insulin is added the fructose

assimilation at a certain plasma concentration is of about the same order as the glucose assimilation at a corresponding glucose concentration in the plasma. If the same quantities of fructose and glucose are added to the blood in a hind-leg preparation the result is the same increase in the fructose and glucose concentrations in the blood. Accordingly fructose, like glucose, distributes itself only to the blood and intercellular fluid, whereas the interior of the muscle cells must be fructose-free. This means that fructose is not transferred "more easily" than glucose from blood to the interior of muscle cells. If we assume that the active link in the transfer of glucose to the interior of the muscle cells, which can be accelerated by insulin, consists in a conversion of the glucose molecule, possibly a phosphorylation, in the surface layer of the muscle cells, it should be possible to conclude from the experiments that the corresponding conversion of the fructose molecule is not affected by insulin.

Summary.

In experiments with artificially perfused hind-leg preparations it is shown that insulin has no effect on the assimilation of fructose in the muscular tissue, whereas the assimilation of glucose is promoted to a considerable extent.

Even in experiments in which the glucose concentration in the blood is artificially reduced to the lowest possible values, insulin has no effect on the fructose assimilation.

The assimilations of glucose and fructose seem to proceed quite independently of each other.

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Studies on the Mutual Effect of Vitamin K and 3,3'-Methylene-bis (4-Hydroxycoumarin) on Prothrombin Formation.

By

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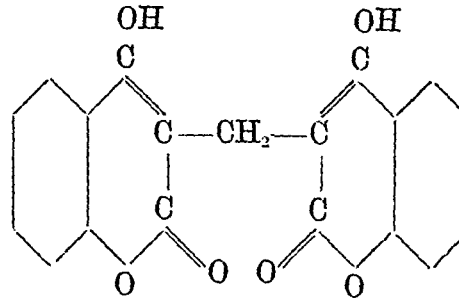
Prothrombin is usually assumed to be formed in the liver, but as yet only little is known as to how this formation takes place. Vitamin K is required for prothrombin formation, but also the mechanism of the action of vitamin K is still obscure, even though it has been established that there is a definite relation between the amount of vitamin K supplied and the amount of prothrombin formed.

In concordance with this, vitamin K deficiency is associated with a decrease in the prothrombin content of the blood. A similar decrease is seen in various infectious and toxic affections of the liver; and in most of these, several aspects of the liver functions will be affected. By fractionated chloroform poisoning in dogs, however, it has been possible to produce a fairly specific inhibition of the prothrombin formation (SMITH, WARNER and BRINKHOUS 1937).

A morbid condition with specific prothrombin deficiency is further found in sweet clover disease (RODERICK 1931), a disease developing in cattle fed spoiled hay of sweet clover (Melilotus alba). Recently it has been possible to isolate and identify the toxic agent in this disease (STAHMAN et al. 1941, CAMPBELL and LINK 1941). Chemically this substance is 3,3'-methylene-bis(4-hydroxycoumarin) which in the following, for the sake of brevity, will be designated dicoumarin.



3,3′-methylene-bis (4-hydroxycoumarin) or "dicoumarin".

As to the action of this substance, it is known to inhibit the prothrombin formation in the liver, but it has not yet been ascertained whether it has a specific inhibitory effect on the function of vitamin K, or whether it exerts a toxic affect on the prothrombin-producing element of the liver.

This question is of importance, not only theoretically but also with a view to the clinical employment of dicoumarin. In the present work, therefore, we have tried to elucidate this question by studies on the effect of simultaneous administration of vitamin K and dicoumarin to animals.

Experimental.

The experiments were carried out on chickens, which are the most suitable animals for the establishment of an alimentary avitaminosis K. For our purpose, a preexisting avitaminosis K (or hypovitaminosis) has proved to be of practical value because it ensures the absence of vitamin K depots, and because the prothrombin level of these animals is low and thus constitutes a sensitive indicator of foreign and inhibitory agencies.

The chickens (white Leghorn), weighing about 100 g, are placed on a vitamin K-free diet, each chicken being kept in a separate cage with a wire-screen floor. In order to prevent coprophagy the cages and food containers were cleaned and sterilized daily. After 3—4 weeks the chickens were vitamin K-deficient — as ascertained by determination of the prothrombin content of the blood. These determinations were carried out partly after Quick's method 1(36), partly after the method given by Dam and Glavind (1938).

For Quick's method a sample of blood (about 1 ml) is withdrawn from the carotid artery into a chilled paraffin-coated tube; 0.9 ml of the blood is mixed with 0.1 ml of 0.1 mol. oxalate solution, and then the plasma is obtained by centrifuging. The thrombokinase preparation here employed has been a watery extract of breast-muscle of fowls, which is stored in ampoules, frozen solid, as given by Dam and Glavind. From this thrombo-

kinase preparation a series of dilutions is made up with physiological salt solution. Detremination of the coagulation is carried out with a dilution, as a rule 10 per cent, that shows optimal activity. The determinations are carried out in a water-bath at 37°. For the test, 0.1 ml oxalate plasma is mixed in a centrifuge tube with 0.1 ml thrombokinase solution. After preliminary heating for 2 min., 0.1 ml 0.04 mol. calcium chloride solution, likewise heated to 37°, is added. The moment of coagulation is reckoned as the point of time when the plasma adheres as a jelly to the bottom of the tube. The interval between the addition of calcium and the moment of coagulation is designated as the prothrombin time.

With this technique a prothrombin time of 47 seconds was found very constantly in chickens on a normal diet, and on every date of the experiment this was confirmed by determination on two control animals. In the chickens on the vitamin K-free diet, after 3—4 weeks, a prothrombin time above 200 seconds was found practically in every test.

A fall in the prothrombin content resulting from vitamin K deficiency or dicoumarin effect will manifest itself by a rise in the prothrombin time. The relation between the prothrombin content of the blood and the prothrombin time has been the object of numerous investigations (e. g. QUICK 1938), showing that with a moderate fall in prothrombin the prothrombin time is prolonged but very little, while with a larger fall it is prolonged at a greatly increased rate.

In a number of experiments the method of DAM and GLAVIND has been employed according to this method, the blood is also withdrawn from a carotid artery into ice-chilled paraffined tubes. The plasma is obtained by centrifuging, and stored at 0°. The thrombokinase preparation is the same as in Quick's method, and a series of dilutions of the thrombokinase is made. The coagulation is determined in micro test tubes at 37°, each tube containing 2 drops of plasma + 2 drops of Ringer's fluid + 1 drop of thrombokinase dilution. From the clotting times obtained by employment of the thrombokinase dilutions, the thrombokinase concentration K which gives a clotting time of 3 min. is calculated by interpolation. If the corresponding concentration for a normal plasma is K_n, the term $K_n/K = 1/R$ is a measure for the prothrombin content of the plasma. 1/R is fairly proportional to the prothrombin content in percent of the normal. So in this way, a relatively slight increase in prothrombin manifests itself more conspiciuously than with Quick's method. On the other hand, K_n varies a good deal from day to day, and this implies a factor of uncertainty.

Experiments on the Effect of Simultaneous Administration of Dicoumarin and Vitamin K.

The rise in prothrombin obtained with a single dose of vitamin K is rather varying, and a more uniform effect is obtained with repeated doses. In the present experiments, therefore, the same dosage was given on three successive days as employed in the biological standardization of vitamin K carried out in this labo-

ratory. Three single doses of 0.00005 mg vitamin K per g of chicken weight is the smallest amount able with certainty to raise the prothrombin content to a normal value in chickens with avitaminosis K.

Also the effect of dicoumarin is variable, and in a more pronounced degree. In the experiments on this problem, too, three daily doses were given; but, as is evident from the curves, it was not practicable in this way entirely to eliminate the individual variation.

For elucidation of the eventual interaction between the effect of vitamin K and that of dicoumarin on the prothrombin, we have carried out a number of experiments with a varying quantitative proportion between the two substances. The vitamin K preparation here employed was 2-methyl-1,4-naphtohydroquinone disulphate.

In the experiments first to be mentioned, the animals were given the same amount of vitamin K and different doses of dicoumarin. The prothrombin content was determined after Quick's method. The experiments were carried out in 4 series with the following dosage of vitamin K: 0.00001, 0.00005, 0.0025 mg per g per day, respectively. The dicoumarin dose varied from 0.005 to 0.5 mg per g per day. The results are recorded in Table 1.

From the preliminary experiments it was known that the smallest dose of dicoumarin (0.00005 mg) here employed with certainty would have an inhibitory effect on the prothrombin formation. As mentioned above, 0.00005 mg of vitamin K is sure to raise the prothrombin level to a normal value. Accordingly, the result of all the above experimental series would have been a normal prothrombin level in series 2—4 if vitamin K had been employed alone, and, conversely, the result would have been a very long prothrombin time in all four experimental series if dicoumarin had been given alone.

Under the given conditions, however, all four series are concordant in showing an increasing prothrombin time, rising from about a normal level with increasing dicoumarin dosage: the smallest dose, 0.00005 mg is nearly or entirely unable to prevent the prothrombin-promoting effect of vitamin K, whereas the largest dose employed, 0.5 mg, is sufficient to overshadow completely the effect of the amount of vitamin K given, even in series 4, in which the vitamin K supply is 500 times the amount required to bring the prothrombin time to a normal level.

Table 1.

Effect of simultaneous administration of vitamin K and dicoumarin.

Series 1. 0.00001 mg. vitamin K per g. chicken weight per day		Series 2. 0.00005 mg. vitamin K per g. chicken weight per day.		Series 3. 0.0025 mg. vitamin K per g. chicken weight per day.		Series 4. 0.025 mg. vitamin K per g. chicken weight per day.	
Dicoumarin mg. (g.) day	Prothrombin time in sec.	Dicoumarin mg. (g.) day	Prothrombin time in sec.	Dicoumarin mg. (g.) day	Prothrombin time in sec.	Dicoumarin mg. (g.) day	Prothrombin time in sec.
0.005	191	0.05	45	0.02	78	0.05	55
0.01	31	0.05	30	0.02	43	0.05	80
0.01	31	0.05	45	0.05	81	0.1	49
0.02	41	0.05	50	0.05	99	0.1	55
0.033	178	0.1	60	0.075	58	0.16	67
0.033	119	0.1	160	0.075	50	0.16	67
0.05	65	0.1	166	0.1	42	0.25	89
0.08	134	0.1	483	0.1	115	0.25	65
0.1	210	0.16	123	0.15	42	0.32	122
0.15	249	0.25	200	0.16	104	0.32	138
0.2	315	0.25	490	0.2	121	0.5	227
		0.25	715	0.25	60	0.5	215
				0.25	104		
				0.28	97		
				0.5	283		
				0.5	540		
				0.5	630		

From this it is evident that the two substances are antagonistic with regard to the prothrombin formation. It is further obvious that in the animals which received a large dose of vitamin K it takes a larger amount of dicoumarin to obtain a distinct prolongation of the prothrombin time. With a decreasing dosage of vitamin K there is a distinct shift of the points to the left. In the two groups of animals which received the largest dose of vitamin K, about 0.5 mg dicoumarin was required in order to eliminate with certainty the effect of vitamin K (prothrombin time 200 sec.), whereas the amount required with the two smallest doses of vitamin K was respectively 0.21 and 0.25 mg. So from this it is evident that the inhibitory effect of vitamin K on dicoumarin increases with its increasing amount.

An increase in the vitamin K dosage thus brings about a stronger attenuation of the effect of dicoumarin, but while the animals in group 4 received 2500 times more vitamin K than the animals in group 1, the amount of dicoumarin required for elimination of the effect of vitamin K is only about 5 times as large. This does not suggest that dicoumarin exerts its effect by displacing vitamin K — for instance, from its place as a component of an enzyme. If so, at any rate, the relation of the two substances is more complicated than the relation between sulfanilamide or similar compounds and p-aminobenzoic acid (WOODS and FILDES 1940), or the relation between (α, γ-dioxy-β, β-dimethylbutyryl)-taurin and panthothenic acid (KUHN et al. 1941), where it is a question of a simple displacement reaction, the result of which can be calculated stoichiometrically.

A characteristic feature of all the series is a considerable dispersion of the values in the range of the smaller dicoumarin doses. As this dispersion is chiefly seen within this range, it may possibly not be attributable to the fact mentioned that the effect of dicoumarin is rather inconstant quantitatively; possibly it is a peculiarity of the field round the point of equilibrium which is most likely to exist between dicoumarin and vitamin K.

In another experimental series performed in order to find such a point of equilibrium, the prothrombin content was determed after DAM and GLAVIND's method. The results are recorded in table 2, in which figures are given for the employed dose of dicoumarin or vitamin K in mg per g per day for three days. The R-values before and after the administration of the two substances respectively are also given. In the first two experiments the dose

of vitamin K was kept constant while that of dicoumarin varied; and in the third experiment the technique was reversed, the dicoumarin dosage being kept constant and the vitamin K dosage varying.

Table 2.

Effect of simultaneous administration of vitamin K and dicoumarin.

First series: 0.00005 mg vitamin K per g of chicken weight per day.

Dicoumarin mg	0.005	0.01	0.02	0.05	0.05
R-value					
before experiment..	5.0	5.7	5.5	5.0	0.6
after experiment...	1.1	10	22	25	0.4

Second series: 0.0002 mg vitamin K per g of chicken weight per day.

Dicoumarin mg	0.002	0.005	0.04	0.05	0.1	0.1	0.16	0.2
R-value								
before experiment..	120	6.7	11	3.3	1.6	1.3	2.6	25
after experiment...	2.6	0.9	2.5	2.8	3.1	15	150	100

Third series: 0.005 mg dicoumarin per g of chicken weight per day.

Vitamin K mg	0	0	0	0.00002	0.00002
	0.00005	0.00008	0.0002	0.0040	
R-value					
before experiment..	9	7	7	10	9.2
	5.0	8	6.9	9	
after experiment...	25	13	11	1.0	9.2
	1.8	0.9	0.9	0.8	

In the first series of experiments the dose of vitamin K was sufficiently large, as mentioned above, to render the prothrombin level normal, i.e. decrease the R-value to less than 2. This was practicable also even with administration of the smallest dose of dicoumarin (0.005 mg), but with all larger doses of dicoumarin this substance got the upper hand.

The second series gave corresponding results. Here the vitamin dose was 4 times higher than in the preceding experiment, and a larger amount of dicoumarin was required in order to make the latter the more effective, namely: at least 0.05—0.1 mg.

In the third series where the dicoumarin dose was kept constant, the first three columns show a fairly constant dicoumarin effect. But even with the smallest dose of vitamin here employed (0.000015 mg) this substance proves the more effective.

All three series of experiments thus show, so to speak, a "neutral point" at which there is a balance between the effect of vitamin K and that of dicoumarin. The quantitative proportion between

the two substances at this point is calculated to be about 1 : 200, 1 : 250, and 1 : 500, as in these series also the absolute amounts are very similar.

Discussion.

From the studies here presented it is evident that dicoumarin and vitamin K have a mutual inhibitory effect on each other. It is further shown that, under certain given conditions, there can be demonstrated a quantitative relation between the two substances, resulting in a mutually inhibitory effect, so that the rate of the prothrombin formation is not influenced by either substance. But these experiments have also shown that this quantitative relation is not constant when the absolute amounts of the substances vary, and that it even varies considerably. An increase or reduction in the dose of dicoumarin given requires a far greater increase or reduction in the dosage of vitamin K if the prothrombin value is to be kept constant. With the smallest active doses the ratio vitamin K/dicoumarin is about 1 : 200—500.

In the literature so far only a few hypotheses have been advanced about the action of dicoumarin. It has been suggested that dicoumarin inhibits the action of vitamin K by a process analogous to the inhibition of the oxidation of succinic acid by malonic acid, pointing out a certain resemblance in the chemical consitution of methyl-naphtoquinone and coumarin (LEHMANN 1942). One of the present writers (JANSEN 1943), on the other hand, considers the dicoumarin effect to be merely toxic — for one thing, because dicoumarin is found to give degenerative changes in the liver, that is, an effect similar to the one produced with fractionated chloroform poisoning.

The results of the present investigation may be said rather to lend support to the latter theory. For when the functional capacity of the liver is lowered by poisoning, it is rather to be expected that the decreased capacity for prothrombin formation to a certain extent may be compensated by an increased supply of vitamin K, whereas a simple stoichiometric relation between the two substances would be expected if the dicoumarin effect resulted from a simple displacement of vitamin K.

As mentioned above, one of the writers (JANSEN 1943) in his experiments with rats, rabbits, and dogs, ascertained injury to the liver parenchyma. In pronounced cases he found fatty degeneration

with central necrosis in a histological picture which greatly resembled that of the liver in chloroform intoxication.

We have investigated whether vitamin K might also have a protecting effect against the injury to the liver produced by dicoumarin. Since OVERMAN et al. (1942) found that, besides vitamin K, also ascorbic acid counteracted the dicoumarin effect, we tried this substance too.

The experiments comprised one group of normal chicks, one group of chicks which received 0.05 mg dicoumarin per g chicken weight per day, one group which received this dose of dicoumarin and in addition the "equivalent" amount of vitamin K (0.000025 mg per g per day), and one group which, in addition to the dicoumarin dose, received ascorbic acid in doses varying from 0.05 to 4 mg per g per day.

The outcome of this experiment, however, was entirely negative: the dose of dicoumarin employed, which can be neutralized by vitamin K, did not result in pathologic-anatomical changes allowing of any comparison of the results; in particular no distinct fatty degeneration or necrotic changes were seen in the liver.

In contrast to OVERMAN, we found no counteracting influence of ascorbic acid on the prothrombin-decreasing effect of dicoumarin — even when given in doses up to 4 mg per g body weight per day.

Nor did ascorbic acid given to chicks with vitamin K deficiency have any effect on the prothrombin level in these animals.

Summary.

In experiments on K-avitaminotic chickens with simultaneous administration of vitamin K and dicoumarin a mutual antagonism is shown to exist between the two substances in the prothrombin formation, and in a certain quantitative proportion this antagonism may eliminate the influence of both substances on the prothrombin formation.

It is further shown that this quantitative relation is not constant, so that a simple stoichiometric relation is out of the question. From this, the conclusion is drawn that the effect of dicoumarin can hardly be a direct inhibition of vitamin K, but is more probably a specific toxic effect which can be compensated in part by the stimulating effect of vitamin K on the prothrombin formation in the liver.

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Further Studies on the Conducting Properties of Human Skin to Direct and Alternating Current.

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Introduction.

In earlier investigations (ROSENDAL 1943) it was shown that the resistance of the skin to direct current (d. c.), just as its resistance to alternating current (a. c.) (ROSENDAL 1940), is localized almost exclusively in the stratum corneum; moreover, that the d. c.-resistance to currents below 0.2 mA/cm² is to a very high degree dependent upon the electrolyte content of this layer and decreases with an increase of the electrolyte content (through moistening of the skin prior to the d. c.-conduction). Further, in the case of skin (7 cm²) moistened with an electrolyte a rise in the d. c.-resistance is observed for anodic and a fall for cathodic conduction[1] when raising the potential from 0.1 to 2—4 volts. This difference in the dependence of the skin resistance upon the two directions of current is evident from the circumstance that the d. c.-resistance of the skin (7 cm²) in the case of anodic conduction, and 2 volts in 2 minutes, may be several times larger than the d. c.-resistance to cathodic conduction, time and voltage being the same. The difference in resistance reaches its maximum after 2—3 minutes conduction in each direction and is highest in the case of potentials of about 2 volts for a skin area of 7 cm². Above 2—4 volts, short-circuiting of the stratum corneum occurs whereby the difference in resistance disappears. Finally, the diffe-

[1] Anodic or cathodic conduction is defined as the d.c.-conduction through the skin with the anode or the cathode, respectively, as different electrode. The indifferent electrode is placed in an electrolyte bath, in which also a large area of the skin, *e. g.*, one arm or a leg, is immersed. Anodic and cathodic resistance are terms applied to the d. c.-resistance to the corresponding direction of current.

rence in resistance is independent of the direction of current with which conduction is started.

Thus *a different conduction for current of low voltage through the stratum corneum in anodic and cathodic conduction is demonstrated.* This difference has been associated with a different permeability to anions and cations through the stratum corneum.

The present paper deals with a further study of this phenomenon, its object being partly, to throw some light on the factors on which the conduction is dependent, and partly, to find an explanation of the phenomenon for the purpose of elucidating the permeability of the stratum corneum and membrane properties.

Method.

Conduction of current. In the determinations of the skin resistance to direct and alternating current the following arrangement was used: A glass cylinder, 2 cm high with an opening 4.9 cm^2 (in some experiments π cm^2) is attached to the volar side of the test person's forearm by means of vaseline and a rubber band. The glass cylinder, the bottom of which is formed by the skin area to be investigated, contains an electrolyte solution. Into this solution dips a polarization-free electrode equipped with a glass tip containing a saturated KCl-solution, solidified by means of 2—3 per cent agar. Then the other forearm, or the leg, of the test subject is immersed in a vessel containing a salt solution (2 per cent) in which a polarization-free electrode is submerged.

Polarization-free electrodes. These are silver-silver chloride electrodes with a saturated solution of potassium chloride, solidified by means of 2—3 per cent agar, of the type described in the previous paper (ROSENDAL 1943) and with the same low resistance, less than 5—10 ohms, and the same low potential, less than 1 mV.

Determination of the d. c.-resistance. This resistance is determined on the basis of voltage and current intensity, alternatingly for anodic and cathodic conduction, in a circuit consisting of the above mentioned object of measurement, a 2 volts accumulator, a commutator, and a light spot galvanometer. The inner resistance of the galvanometer is 16.79 ohms, and the sensitivity is $1° = 0.182 \times 10^{-6}$ amperes. By means of shunt resistances the sensitivity can be varied between $1° = 0.226 \times 10^{-6}$ amperes and $1° = 2\,260 \times 10^{-6}$ amperes. The calculated d. c.-resistance now applies to the composite object of measurement: Electrode — electrolyte — 4.9 cm^2 of skin — internal tissue — submerged extremity — salt solution — electrode. As shown in the previous paper, however, the d. c.-resistance is almost solely determined by the small skin area of 4.9 cm^2, so that the set-up described permits an investigation of the d. c.-resistance to anodic and cathodic conduction for the skin area in question. As mentioned, it is especially the difference in the resistance to the 2 directions of current that throws

light on the question of the different conduction through the stratum corneum. In the main part of the investigations reported in the present paper this quantity is derived from the formula: Percentage resistance difference $= \frac{(\text{anodic resistance} - \text{cathodic resistance}) \times 100}{\text{cathodic resistance}}$. The error in the determination of the resistance is less than 1 per cent.

Measuring the potential. In the investigations of the voltage across the stratum corneum during and after anodic and cathodic d. c.-conduction, measurements have been made of the potential between the two polarization-free electrodes after switching off the d. c. Voltage measurements have moreover been made during and after anodic and cathodic d. c.-conduction by means of special electrodes, arranged as shown in fig. 1: I) An electrode dipping below the surface of the electrolyte solution in the glass vessel attached to the volar side of the forearm, and an electrode close to the surface of the skin at the bottom of the vessel. II) An electrode in the electrolyte solution, and an electrode penetrating the stratum corneum and reaching down into the subcutaneous layer, just outside the glass cylinder. III) An electrode in the electrolyte solution, and one placed in the stratum corneum, corresponding to the skin area investigated.

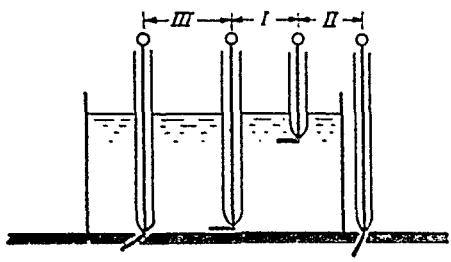

Fig. 1. Arrangement of the iron electrodes in the electrolyte solution (I), in the corium outside the glass tube (II) and in the stratum corneum inside the glass tube (III).

The following electrode pairs are used: a) Ag-AgCl electrode with a KCl-solution, of the type employed as reference electrode in pH determinations by means of a valve potentiometer. b) Ag-AgCl electrodes. c) Iron electrodes. d) Copper electrodes. Electrodes b, c and d consist of 1/3—1/2 mm thick metal wire, fused into a glass tube with angularly bent tip, the wire extending $^1/_2$ cm out of the glass tube. The electrode pairs a, b, c and d are used in experiments of type I, while electrodepair c is used in experiments of types II and III.

The voltage is measured by means of a valve potentiometer which permits a determination of up to 1 000 mV, with an error of about 1/2 mV.

Determination of a. c.-resistance and phase angle.[1] The impedance (Z) and the phase angle (φ) of the object of measurement are determined at different frequencies by means of a GRÜTZMACHER bridge. The method is described by GRÜTZMACHER (1934) and by NIELSEN (1936). The normal resistance is an inductance-free resistance decade of 100 000 ohms, with an error of 1 per cent. An a. c.-valve voltmeter is used for

[1] This part of the investigation was carried out at the Finsen Laboratory in Copenhagen, and the author is greatly indebted to the Physician-in-Chief, O. M. HENRIQUES, D. Sc., for permission to perform this work.

measuring voltage. The source of current is a hetero-dyne oscillator with a frequency range from 100 to 40 000 cycles and a voltage range up to 50 volts. The object of measurement is connected to the bridge arrangement, as described on p. 184, and screened wires are used.

From the impedance measured (Z) (error 1 per cent), and the phase angle (φ) (error $< 1/4°$) it is possible by means of the equations $R_s = Z \cdot \cos\varphi$ an $X_s = Z \cdot \sin\varphi$ to calculate the equivalent serial resistance (R_s) and serial reactance (X_s) components, and from $R_p = \frac{Z}{\cos\varphi}$ as well as $X_p = \frac{Z}{\sin\varphi}$, the corresponding components for a parallel system.

Experimental Results.

A. Investigations on Anodic and Cathodic d.c.-Conduction through the Skin.

1) *Individual, regional and daily variation in the resistance of the stratum corneum to anodic and cathodic conduction, and of the percentage resistance difference* (*see p. 185*).

The results recorded in table I show the individual and regional variation as well as the daily variation in the resistance of the stratum corneum to the 2 directions of current, and the variations in the percentage resistance difference. The table gives the resistance to anodic and cathodic d. c.-conduction, and also the percentage resistance difference in the case of three test persons, for different regions of the volar side of the person's arm and, finally, in two test persons for the same region of measurement over a period of several days.

The table shows the great variation in the resistance to both directions of current and in the percentage resistance difference in the case of different persons, different places on the arm, and for the same region of measurement on different days. Hence, the following investigations are, in each experimental series, carried out on the same person, the same region, and on the same day.

2) *The significance of various inorganic electrolytes with respect to the d. c.-resistance of the skin to the 2 directions of current and with regard to the percentage resistance difference.*

a) Monovalent electrolytes.

In order to investigate the significance of some monovalent electrolytes with respect to the d. c.-resistance of the stratum corneum to the 2 directions of current, determinations have been

Table I.

Individual, regional and daily variation of the d.c.-resistance in ohms and of the percentage resistance difference for 4.9 cm² skin — 2 volts, electrolyte 1 molar KCl solution, anodic and cathodic conduction 2 minutes in each direction and after conduction for 6 min.

	Individual variation			Regional variation			
Test person Region: forearm	H. H.	H. F.	P. W.	P. W. upper arm	forearm near the elbow	middle of forearm	proximal to wrist
Anodic resistance	475,000	178,000	95,000	5,950	95,000	72,000	1,920
Cathodic resistance	90,000	66,000	65,000	3,250	65,000	45,000	1,440
Percentage resistance difference	428	170	46	83	46	60	33

	Daily variation									
Test person Region: forearm	H. H.					H. F.				
Date	10/9	11/9	12/9	14/9	15/9	10/9	11/9	12/9	14/9	15/9
Anodic resistance	475,000	142,000	325,000	79,500	135,000	178,000	119,000	67,500	105,000	216,000
Cathodic resistance	90,000	45,000	103,000	22,000	25,500	66,000	30,000	13,000	27,000	130,000
Percentage resistance difference	428	216	216	262	430	170	297	420	289	66

made of the d. c.-resistance to anodic and cathodic conduction, using solutions of NH_4Cl, NaCl, KCl, CsCl, KBr as well as KJ as contact electrolyte. In the individual series either the cation or the anion has been varied, maintaining the same anion (Cl^-) and cation (K^+) respectively. The skin is washed several times with distilled water between each determination of its resistance. The results in the case of 3 persons are given in table II.

Table II.

Resistance in ohms to anodic and cathodic conduction, each lasting 2 min. 4.9 cm^2 skin (forearm) and 2 volts and the percentage resistance difference after moistening the skin and conduction for 5—10 min., using different salts as contact electrolyte. The proportionality factors of the table are calculated on the basis of the percentage resistance difference, the first value in each set of experiments being put = 100.

Test person	Electrolyte (m = molar)	Anodic resist.	Cathodic resist.	Percentage resistance difference	Proportionality factor
H.F.	1/10 m KCl	66,500	43,500	112	100
—	— — KBr	32,500	16,200	100	89
—	— — KJ	28,700	15,500	85	76
H.F.	1/1 m KJ	43,000	9,300	362	100
—	— — KCl	26,500	10,700	148	41
H.H.	1/10 m KJ	442,000	330,000	34	100
—	— — KBr	530,000	400,000	22	66
—	— — KCl	520 000	400 000	30	88
H.H.	1/1 m KJ	115,000	34,500	232	100
—	— — KCl	89,000	34,250	160	69
—	— — KJ	81,000	35,000	131	56
H.H.	1/1 m NH_4Cl	177,000	100,000	77	
—	— — NaCl	96,000	56,000	71	
J.H.	1/1 m KCl	176,000	30,000	487	
—	— — CsCl	200,000	42,000	376	

From the calculated percentage resistance differences and the corresponding proportionality factors it will be seen that the resistance to the 2 directions of current is independent of whether the contact electrolyte is a solution of KCl, KBr or KJ, conformable results being obtained when the sequence of the 3 salts is changed. The same is also true of solutions of NH_4Cl and CsCl.

Assuming that the d. c. in the case of anodic conduction is conveyed to the stratum corneum by means of the cation, and in the

case of cathodic conduction by the anion, the experiments show no interdependence of the resistance of the stratum corneum to anodic conduction and the cations used, nor of the resistance to cathodic conduction and the anions used.

b) *Concentration of electrolyte.*

When investigating the dependence of the skin resistance and of the percentage resistance difference upon the concentration of the electrolyte, the contact electrolyte consists of aqueous solutions of KNO_3, KCl, K_2SO_4, NH_4Cl, $FeCl_3$ and $AlCl_3$. In some of the experiments the concentration is increased from 1/100 to 1/10 molar, in others from 1/10 to 1 molar, while in some even to the point of saturation. The individual sets of experiments are, as previously mentioned, applied to different regions, and the skin is washed several times with the salt solution in question before determining the resistance. The results are recorded in table III and fig. 2.

Table III.

Resistance in ohms to anodic and cathodic conduction, each lasting 2 min. 4.9 cm² skin (forearm) 2 volts, and the percentage resistance difference after moistening and conduction for 5—10 min., with different salts of different concentrations as contact electrolyte.

Test person	Electrolyte (m = molar)	Anodic resist.	Cathodic resist.	Percentage resistance difference
H.F.	1/10 m KNO_3	119,000	79,500	49
—	1/1 — —	41,000	22,000	86
P.B.	1/2 m KCl	44,000	20,000	120
—	saturated —	22,500	6,300	257
P.B.	1/10 m K_2SO_4	65,000	28,000	132
—	saturated —	29,000	9,000	222
P.B.	1/10 m NH_4Cl	26,000	16,000	62
—	1/1 — —	12,600	4,500	180
H.F.	1/100 m $FeCl_3$	900,000	110,000	718
—	1/50 — —	395,000	31,500	1,150
H.H.	1/100 m $AlCl_3$	750,000	103,000	629
—	1/10 — —	500,000	12,500	3,900

In all experiments a fall occurs in the resistance to the cathodic as well as to the anodic conduction, and a rise in the percentage

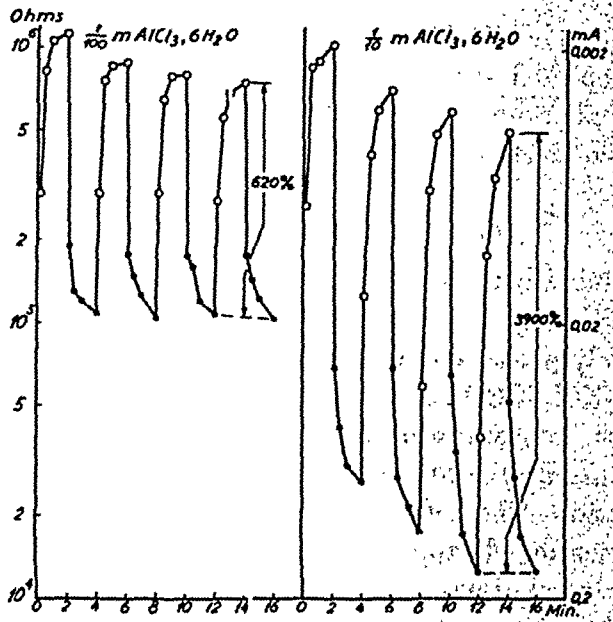

Fig. 2. The significance of the concentration of the contact electrolyte to the resistance of the stratum corneum to alternatingly anodic and cathodic d. c.-conduction (2 V.) Region of measurement 4.9 cm² on the volar side of the forearm Test person H. H.

Ordinate: (left) resistance in ohms, (right) current intensity in mA.
Abscissae: time in minutes.
○ anodic conduction, ● cathodic conduction.

resistance difference, when the concentration of the contact electrolyte is increased. The curves of fig. 2 show that the resistance to anodic conduction is independent of the rise in the electrolyte concentration. The same is not true of the resistance to cathodic conduction, which decreases considerably at high concentrations, with a corresponding rise in the percentage resistance difference. The smooth fall in the resistance to anodic conduction in the case of both concentrations is, as previously mentioned, due to a rise in the electrolyte content of the stratum corneum owing to the moistening and the conduction of the electric current. As regards the fall in the resistance to cathodic conduction at the high concentration of electrolyte, it is natural to associate this phenomenon with the rise in the concentration of anions that convey the current into the skin by cathodic conduction, and to which, one may suppose, the stratum corneum is most permeable.

c) *The valency of the electrolyte.*

An investigation has been made of the influence which the valency, and especially that of the cation, has on the d. c.-resistance

of the stratum corneum to the 2 directions of current and on the percentage resistance difference. Two test persons are included and a determination is made of the d. c.-resistance to anodic and cathodic conduction, using equimolar salt solutions with increasing valency as contact electrolyte. In the individual experiments the resistance is determined before and after exchange of equimolar solutions of $FeCl_2$ with $FeCl_3$, KCl with $FeCl_3$, KCl with $AlCl_3$, and KCl with $CaCl_2$ and then with $AlCl_3$. The skin is washed several times with the salt solution used before the resistance is determined.

Figs. 3, 4 and 5 show the resistance of the stratum corneum to the 2 directions of current, as well as the calculated percentage resistance difference in the case of the 2 test persons, the contact electrolyte being, respectively, 1/10 molar $FeCl_2$, $4H_2O$ and 1/10 molar $FeCl_3$, $6H_2O$, 1/10 molar KCl and 1/10 molar $AlCl_3$, $6H_2O$, 1 molar KCl, 1 molar $CaCl_2$ and 1 molar $AlCl_3$, $6H_2O$.

The curves show that the resistance to anodic conduction is at about the same level before and after the rise in the valency of the cation. The resistance falls slightly due to the moistening of the stratum corneum with the electrolyte. Contrarily it is found that the resistance to cathodic conduction falls very considerably with a corresponding rise in the percentage resistance difference.

Thus the experiments give results which resemble those obtained when increasing the concentration of the salt solution (section b, p. 189), *i. e.*, a large fall in the resistance to cathodic conduction with practically no changed resistance to anodic conduction. The explanation of the fall in resistance should therefore be explainable by *the rise in the anion concentration* (Cl^-) *which is brought about by the higher cation valency.*

Since the flow of current, as previously mentioned, in connection with the conduction through the stratum corneum, must be assumed to take place by means of the inorganic cations, and in the case of cathodic conduction by the inorganic anions, this experiment, too, makes it probable *that the stratum corneum is more permeable to inorganic anions than to inorganic cations.*

3) *The significance of some organic electrolytes with respect to the d. c.-resistance of the stratum corneum and the percentage resistance difference.*

Having shown that the resistance of the stratum corneum to cathodic conduction decreases with a rising concentration of the

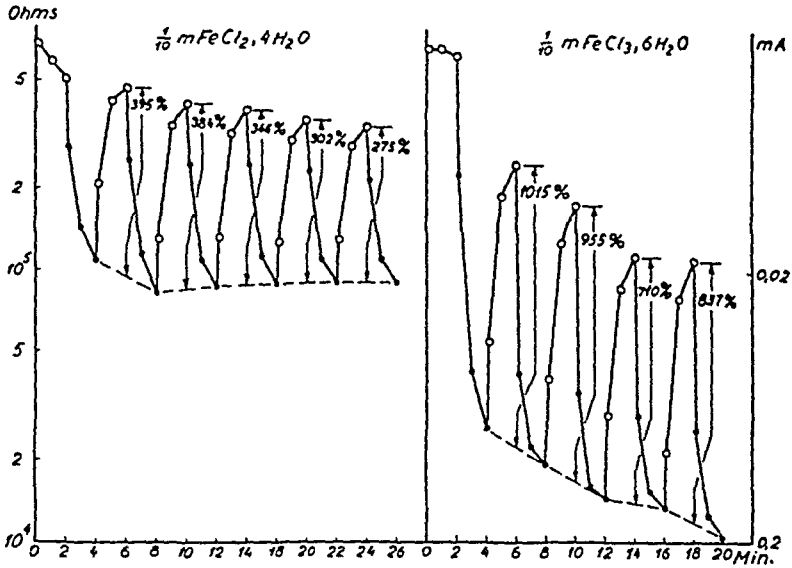
Ohms
$\frac{1}{10}$ mFeCl$_2$, 4H$_2$O
$\frac{1}{10}$ mFeCl$_3$, 6H$_2$O
mA
395 %
384 %
344 %
302 %
275 %
1015 %
955 %
810 %
837 %
0,02
0,2
Min.

Fig. 3.

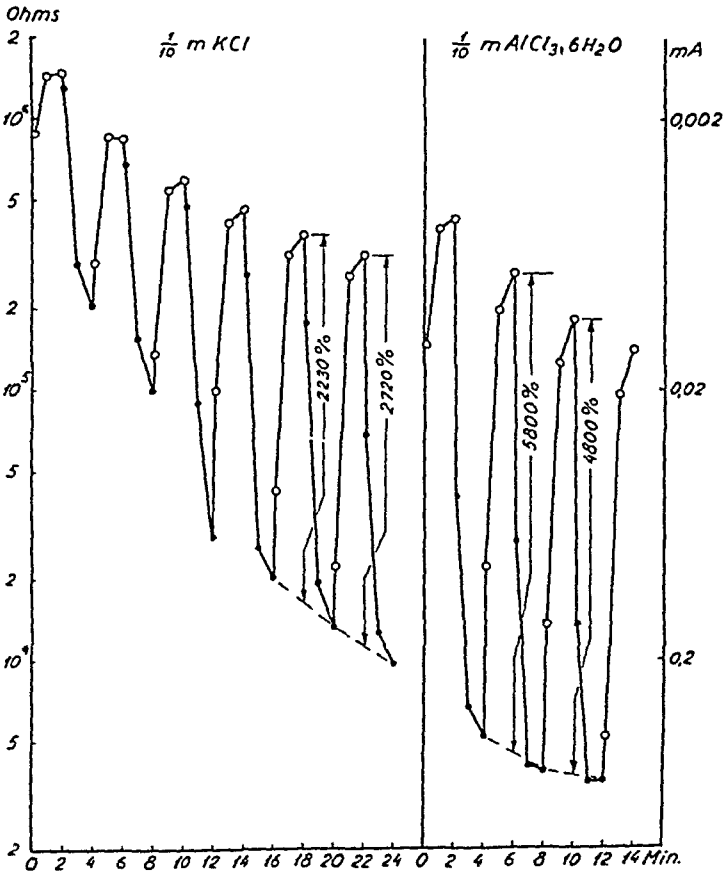
Ohms
$\frac{1}{10}$ m KCl
$\frac{1}{10}$ mAlCl$_3$, 6H$_2$O
mA
2230 %
2720 %
5800 %
4800 %
0,002
0,02
0,2
Min.

Fig. 4.

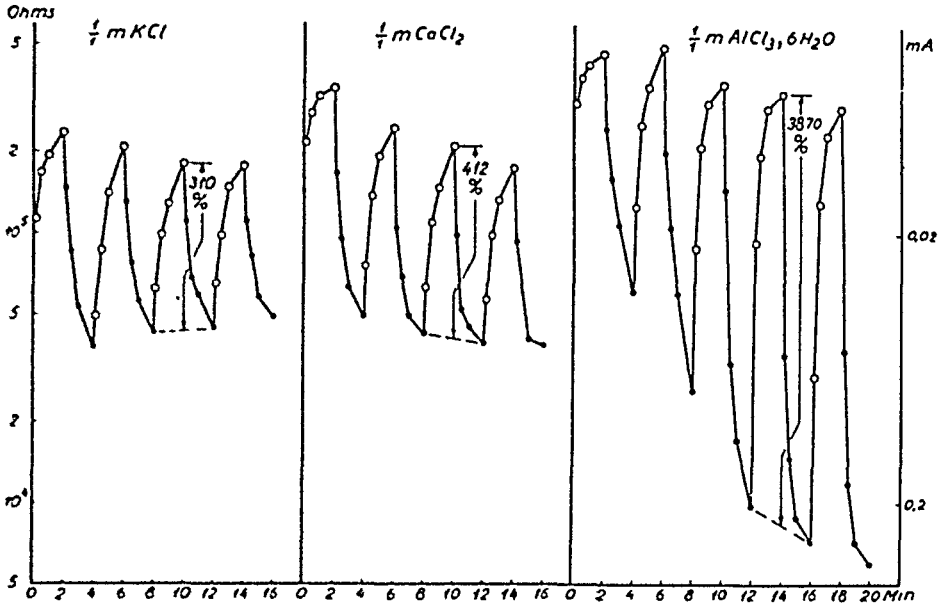

Fig. 5.

Figs. 3, 4, 5. The significance of the valency of the contact electrolyte, especially the cation, to the resistance to alternatingly anodic and cathodic d. c.-conduction for 2 minutes at 2 volts. 4.9 cm² of the stratum corneum on the volar side of the forearm.

Test person: Fig. 3, H. F. Figs. 4 and 5, H. F and H. H.
Ordinates (left) resistance in ohms, (right) current intensity in mA.
Abscissae: time in minutes.
○ anodic conduction, ● cathodic conduction.

inorganic anion (Cl^-), we shall now investigate whether the size of the anion has any influence upon the resistance of the stratum corneum to the same direction of current. Hence, determinations are made of the resistance to the 2 directions of current, using solutions of 1/2 and 1 molar sodium citrate, 1 molar sodium benzoate, and 1/10 molar sodium taurocholate as contact electrolyte. The solutions all have a large organic anion and the same inorganic cation.

The results of experiments on 4 test persons may be summed up as follows: The difference in the resistance of the stratum corneum to the 2 directions of current, employing the above mentioned organic electrolyte solutions, is considerably smaller than when inorganic salt solutions are used as contact electrolyte, the percentage resistance difference varying between 5 and 15 per cent. Some of the experiments even show that the resistance to cathodic conduction is greater than to anodic conduction.

These results are best explained by assuming that the permeability of stratum corneum to organic anions in cathodic conduc-

tion is smaller than that demonstrated in the earlier sections in the case of inorganic anions, thus indicating that the size of the anion has an influence on the d. c.-resistance of the skin.

When considering the reason for the above demonstrated differences in the resistance of the stratum corneum to the 2 directions of current when inorganic salt solutions are used as electrolyte, one may seek the explanation either in electrolytic polarization as occurring only in the case of anodic conduction, or in a different conductivity of the stratum corneum for the 2 directions of current. Some supplementary investigations have been carried out.

First, measurements have been made of the voltage across the stratum corneum before, during and after anodic and cathodic conduction, in order to determine whether, after switching off the d. c. one can measure a potential of the same magnitude and with the same sign as the voltage applied.

Next, measurements have been made of the a. c.-resistance of the stratum corneum immediately after anodic or cathodic d.c.-conduction, for the purpose of investigating whether the a. c.-resistance of the stratum corneum changes parallel to the d. c.-resistance to the 2 directions of current, as one would expect if the second of the above explanations were correct.[1]

The results of these measurements are reported in the following two sections.

B. Investigations on the Potential across the Stratum Corneum during and after Anodic and Cathodic d.c.-Conduction.

The technique mentioned on p. 185 has been employed in a series of experiments involving 2 test persons, for the purpose of recording the voltage across the stratum corneum before, during and after anodic and cathodic d. c.-conduction. The results are as follows:

1) By conduction from the two current-supplying, polarization-free Ag-AgCl-KCl-electrodes to the valve potentiometer a poten-

[1] In the case of a polarization cell consisting of an Ag- and an AgCl-electrode in a solution of KCl we find for the d. c.-conduction from the AgCl-electrode through the KCl-solution to the Ag-electrode an increasing resistance like the change in resistance of the stratum corneum during anodic conduction. After switching off the d. c. a polarization potential can be measured across the cell, of almost the same magnitude and with the same sign as the potential applied. The a. c.-resistance of the polarization cell is constant and *does not change* parallel to the d. c.-resistance.

tial of not more than 20 mV between the electrodes can be recorded after discontinuing the 2 000 mV applied across the object of measurement in the case of anodic conduction.

2) By measuring with two special electrodes (type a, b, c and d, p. 185) placed, respectively, on the surface and deep in an electrolyte (the solution being 1/10 molar, 1 molar or saturated KCl, 1/10 molar $CuSO_4$, and 1/10 molar $FeCl_2$) in the glass tube attached to the skin (Fig. 1, I, p. 185), there can be recorded a potential of not more than 20 mV during anodic and cathodic conduction with 1 000 mV and with a typical change in the resistance of stratum corneum. After switching off the current the potential is less than 10 mV.

3) By measuring the potential between an iron electrode (type c, p. 185) in a 1/10 molar $FeCl_2$, $4H_2O$ solution in the glass tube attached to the skin and an iron electrode penetrating into the subcutaneous tissue just outside the glass tube (fig. 1, II, p. 185) during anodic and cathodic conduction the potential is found to be almost of the same order of magnitude as that applied across the object of measurement (1 000 mV), and it is constant in spite of typical changes in d. c.-resistance to the 2 directions of current. After stopping both the anodic and the cathodic conduction no potential can be recorded between the iron electrodes.

4) Finally, measurements have been made of the potential between an iron electrode in a 1/10 molar $FeCl_2$, $4H_2O$ solution in the glass tube attached to the skin and an iron electrode introduced into the stratum corneum within the glass tube (fig. 1, III, p. 185) during and after d. c.-conduction with a potential of 900—1 000 mV. In all experiments it is possible to record a part of the applied voltage, and the major part during anodic conduction. Some of the experiments show parallelism between the recorded voltage and the magnitude of the resistance of stratum corneum to the 2 directions of current. After discontinuing the anodic conduction (900—1 000 mV) it is only possible to record a potential between the iron electrodes of 50—100 mV, with the same sign as the voltage applied.[1]

[1] The recording of the voltage across the iron electrodes, and of the a. c.-resistance (following section) after anodic and cathodic conduction is made 5—10 seconds after switching off the d. c. It has been found, however, that the spontaneous change in the d. c.-resistance to anodic and cathodic conduction *after* stopping the current is so slow that no error is committed by comparing the measured d. c.-resistance to the 2 directions of current with the potential between the iron electrodes measured 5—10 seconds later (or with the a. c.-resistance considered in the next section).

The different voltage recordings show that no potential can be measured across the stratum corneum which can explain the increase in resistance during anodic conduction.

The small potential measured in experiments of type 2 corresponds to the slight fall in voltage in the good conducting electrolyte in the glass tube attached to the skin. The agreement found between the potential measured across the stratum corneum in experiments of type 3 and the voltage applied across the object of measurement shows that the major part of the fall in potential occurs through the stratum corneum, which is also the seat of the resistance of the object of measurement. The voltage measurements in experiments of type 4 must be accepted with a certain reservation, since the introduction of the iron electrode into the stratum corneum alone is difficult, and it is probable that the tip of this electrode extends down into the stratum germinativum (fig. 1, III, p. 185). This method involves both tapping of voltage in the poorly conducting stratum corneum and in the good conducting stratum germinativum, with a possibility of secondary currents between these 2 places of the iron electrode — a circumstance which perhaps may explain the potential of 50—100 mV measured in the experiments. When we find the potential measured between the iron electrodes to change parallel to the resistance during anodic and cathodic conduction — and the change being most considerable during the anodic conduction — the explanation must be that the 2 electrodes are tapping part of the voltage applied across the stratum corneum, and that this part is at its greatest when the resistance between the 2 iron electrodes is maximal.

Summing up the experimental results it will be seen that *the demonstrated difference in the resistances of the stratum corneum cannot be explained by electrolytic polarization.*

C. The a.c.-Resistance of the Skin after Anodic and Cathodic d.c.-Conduction.

Using the arrangement devised by GRÜTZMACHER (p. 185), a number of determinations have been made of the stratum corneum's a. c.-resistance, or impedance and phase angle at 200 and 2 000 cycles, 5—10 seconds after measuring the d. c.-resistance to anodic and cathodic d. c.-conduction respectively. The results are given in table IV.

Table IV.

D.c.-resistance in ohms for 4.9 cm^2 on the volar side of the forearm — the potential being 2 volts and the contact electrolyte a saturated solution of KCl — after anodic and cathodic conduction respectively, and the impedance (Z) in ohms and the phase angle (φ) in degrees, together with the calculated values of R_p and X_p in ohms determined 5—10 seconds after switching off the d.c.-current. Test person K.C. (Cycle = c).

Direction of current	Resistance ohms	$Z_{200\ c.}$ ohms	$\varphi_{200\ c.}$ degrees	$Z_{2000\ c.}$ ohms	$\varphi_{2000\ c.}$ degrees	R_p ohms	X_p ohms
anodic . .	57,000	16,500	67.8			43,700	17,800
cathodic .	20,000	13,400	49			20,500	17,800
anodic . .	57,000	16,200	65			38,400	17,900
anodic . .	57,000			2,340	78,2	11,450	2,390
cathodic .	17,400			2,330	74	8,460	2,420
anodic . .	50,000			2,340	78	11,250	2,390
cathodic .	15,400			2,330	74	8,460	2,420

The table shows that the a. c.-resistance and the phase angle of stratum corneum at 200 and 2 000 cycles change parallel to the d. c.-resistance to the 2 directions of current. From the calculated equivalent resistance and reactance components R_p and X_p (see p. 186) for a parallel system for the stratum corneum it will be seen that it is solely the ohmic component of the a. c.-resistance that is changed by anodic and cathodic conduction, while the reactance X_p remains constant. The change in R_p becomes more prominent at 200 than at 2 000 cycles, in agreement with the circumstance that the a. c.-conduction through the stratum corneum at low frequencies predominantly occurs through the ohmic component, while the conduction at higher frequencies primarily is capacitive.

If we now imagine the ohmic component of the a. c.-resistance localized to the stratum corneum, it must be to the same more or less electrolytefilled conduits or spaces that also serve in the d. c.-conduction for anodic and cathodic direction of the current. Hence, when it is possible to show a change in a. c.-resistance of the stratum corneum parallel to the d. c.-resistance in the case of anodic and cathodic conduction *it must mean that the different d. c.-resistance is due to a different conductivity in stratum corneum for the 2 directions of current.*

In addition to the above mentioned investigations on the a. c.-resistance of stratum corneum after anodic and cathodic d. c.-conduction, determinations have also been made of the a. c.-resistance for the same object of measurement at 200 and 2 000 cycles, before and after d. c.-conduction for a certain period of time, the electrolyte being a 1 molar and a saturated KCl-solution respectively. This investigation too shows a fall in the a. c.-resistance of the stratum corneum, and only in the ohmic component, parallel to the fall in the d. c.-resistance, the fall likewise being due to a change in the conductivity of stratum corneum, corresponding to the rise in the electrolyte content of stratum corneum which is demonstrated in an earlier paper (ROSENDAL 1943). The fall in the a. c.-resistance is most considerable when the saturated KCl-solution is used as electrolyte. Moreover, it is greater at 200 than at 2 000 cycles, and non-existent at 20 000 cycles — in agreement with what has previously been mentioned (p. 197) regarding the a.c.-conduction through the stratum corneum at low and high frequencies.

An investigation of the test object's a. c.-resistance at 200, 2 000 and 20 000 cycles shows that the a. c.-resistance at these frequencies is independent of the a. c.-potential within the range from 50 to 5 000 mV (no higher voltage has been tried). Since the earlier investigations on the conducting properties of the human organism to alternating current (ROSENDAL 1940) all are carried out at 50 mV, their scope was limited. The present experimental results show, however, that we are now able to extend the validity of the earlier a. c.-investigations up to a. c.-potentials of as much as 5 000 mV. The author has in mind, especially, the demonstrated frequency dependence applying to frequencies above 500—1 000 cycles for the a. c.-resistance (Z) of stratum corneum, and the frequency independence applying to the phase angle (φ) of stratum corneum, expressed by the equations $Z = k \cdot \omega^{-a}$ and $\varphi = a \cdot \frac{\pi}{2}$ where ω is the angular velocity and $a = 0.9$.

Discussion.

The present investigations show that the difference in the resistance of the stratum corneum to anodic and cathodic d. c.-conduction is not due to electrolytic polarization in the case of the anodic conduction, but is caused by a difference in the con-

ductivity of the stratum corneum with respect to the 2 directions of current.

Theoretically, the d. c.-conduction through the corneous layer must be determined by the permeability to anions and cations which are present in the solution on the outside surface of the stratum corneum and in the tissue fluid on the inside of the corneus layer. The experiments show, however, that the difference in resistance to the 2 directions of current to a very high degree is determined by the nature and the concentration of the salt solution on the surface of the stratum corneum, and, especially, that the percentage resistance difference increases with a rise in the concentration of inorganic anion. If the contact solution applied to the stratum corneum is free from electrolyte, or contains but little electrolyte (distilled water has been tried) then the difference in resistance to the 2 directions of current disappears or falls below 10 per cent, showing that the electrolytes of the tissue fluid do not contribute to the difference in resistance. An experiment with tissue fluid from a blister as contact electrolyte on the corneus layer shows a difference in the resistance to the 2 directions of current similar to that found in the case of inorganic salt solutions. Similar results are obtained in the case of the stratum corneum from a blister and from an amputated leg, with the same salt solution on each side of the corneous layer. Considering these experimental results it will be seen that the difference in the resistance of the stratum corneum to the 2 directions of current is not due to any difference in the electrolyte solution on the outside and on the inside of the stratum corneum. The only explanation is *that the stratum corneum is permeable to inorganic anions, but not, or at least only to a slight degree, to inorganic cations.*

When the corneous layer is more permeable to inorganic anions than to inorganic cations it cannot be because of a difference in the ionequivalent electric conductivity, since this is practically the same for the anions and cations used. Nor can the reason be the different size of inorganic anions and cations; in the case of the ions used here roentgen spectrography shows a diameter of 1—2 Å[1], and about 3 Å if we include the water of hydration in the case of Fe^{++}, Fe^{+++} and Al^{+++}.

As an explanation of the difference in the permeability to inorganic anions and cations through the stratum corneum one might

[1] Internat. Tabelle zur Bestimmung von Kristallstrukturen, Vol. II, Berlin 1935.

assume that the corneous layer is an electropositively charged membrane, and hence only permeable to inorganic anions.

A basis for this assumption is found in the investigations by BETHE and TOROPOFF (1914 and 1915), MICHAELIS and co-workers (1925—1927), MOND and HOFFMANN (1928), FREUNDLICH (1930) and HÖBER (1936), all dealing with the polarity of organic membranes. According to these investigations the electronegatively charged collodion membrane is permeable to cations, but not to anions. If the pH of the membrane is lowered, *i. e.*, the membrane becomes electropositively charged, the permeability will change and the membrane is permeable to anions only. When d. c. is conducted through a collodion membrane, with equimolar electrolyte solutions on either side, it may show unipolar conductivity, *i. e.*, function as a rectifier (LABES and ZAIN (1927), BLINKS (1930). The change in the conductivity is due to a change in ohmic resistance, and back-e. m. f. is of less importance.

If the protein membrane "stratum corneum" has a positive wall charge in all its pores — which according to the above mentioned investigations should be true of a protein membrane at higher hydrogen ion concentrations — then we can explain why the conductivity is higher for the inorganic anion than for the inorganic cation.

In support of the idea of the higher hydrogen ion concentration in the corneous layer reference may be made to SCHADE and MARCHIONINI (1928) who found acid reaction on the surface of the stratum corneum.

When organic salt solutions are used as contact electrolyte there occurs a reduction of the percentage resistance difference, which indicates that the size of the anion has an influence on the permeability. Finally, it has been found in experiments with HCl-solutions of different concentrations, up to 1/10 N, that the resistance of the stratum corneum to anodic conduction is lower than it is to cathodic conduction. Since the transport of the current into the stratum corneum during anodic conduction must be supposed to take place by means of hydrogen ions, and since the equivalent conductivity of these ions is several times larger than that of the chloride ions (315—64), we may here have an explanation of the reason for the change in the conductivity of the stratum corneum.

The different conductivity for the 2 directions of current is, as mentioned in the earlier paper, dependent on the size of the

potential difference. The difference is not found at low potentials (up to 100 mV), and it likewise disappears when the potential exceeds 2—4 volts, when there occurs a shortcircuiting of the stratum corneum, setting up low-ohmic shunt resistances. The difference in conductivity for the 2 directions of current is most pronounced when the object of measurement is a small region of skin in series with a many times larger indifferent skin area. If the object of measurement involves 2 skin areas of equal size, the particular permeabilities will neutralize each other.

According to the earlier theory regarding the conducting properties of the skin to electric current — as it is presented, for example, in SCHAEFER's "Electrophysiologie" (1940) — the high-frequent a. c.-resistance is the true resistance of the skin, while the d. c.-resistance must be considered a polarization phenomenon, localized to cell membranes in the living cell layer of the stratum germinativum. The stratum corneum is thought to be without any significance for the resistance in comparison with the stratum germinativum. A change in the membrane permeability of the living cell layer should be recorded as a change in the d. c.-resistance. According to the present investigations, it becomes necessary to abandon this theory. The d. c.-resistance and the a. c.-resistance represent two different aspects of the corneus layer as electric conductor, determined, respectively, by the electrolyte content in and on the stratum corneum, as well as by the direction of current, and the dielectric effect of the stratum corneum. A change in the electrolyte content can be recorded both as a change in the d. c.- and the a. c.-resistance, as it has been shown in the case of Graves' disease and myxedema.

A later paper will deal with further investigations on the conduction of alternating current through the stratum corneum, and especially with the dielectric properties of this layer.

Summary.

The resistance of 4.9 cm^2 of the stratum corneum to direct current (2 volts), when inorganic salt solution is used as contact electrolyte, is found to be lower at cathodic conduction[1] where inorganic anions pass into the stratum corneum than at anodic conduction[1] where inorganic cations pass into the corneous layer.

A rise in the concentration of the contact electrolyte, or in the valency of the cations — both changes producing an increase in the

[1] See foot-note p. 183.

concentration of the inorganic anions of the contact electrolyte — leads to a further reduction of the resistance at cathodic conduction, while the resistance at anodic conduction is not changed.

Measurements of the potential across the stratum corneum before, during and after conduction of direct current, with anodic and cathodic direction, indicate that the greater resistance to anodic conduction is not due to electrolytic polarization.

Determination of the a. c.-resistance at 200 and 2 000 cycles after anodic and cathodic d. c.-conduction shows that the difference in resistance to the two directions of current is due to a different conductivity of the stratum corneum. The different conductivity is caused by the fact that the stratum corneum is more permeable to inorganic anions than to inorganic cations. It is assumed that the reason is that the stratum corneum, like other organic membranes, is positively charged.

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On the Occurrence of a Thiamin Inactivating Factor in Some Species of Swedish Fish.

By

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The disease of the fox commonly known as Chastek paralysis represents an unusual type of a vitamin deficiency disease in that it results from a destruction of the dietary thiamin occurring, preliminary to absorbtion. The exhaustive studies of GREEN, CARLSSON and EVANS (1941) clearly demonstrated that the destruction, and consequently also the paralysis, occurred if certain species of fresh or frozen fish were added to the ration given to foxes. Since the addition of excessive amounts of thiamin to a ration containing fish prevented the disease, it was concluded that some component of the fish was responsible for the thiamin loss and further that this loss occurred during the intimate mixing of the fish tissue with the thiamin containing portions of the diet. WOLLEY (1941) reported that the active ingredient in fresh carp was heat labile, not dialysable and could be extracted with 10 per cent solution of sodium chloride. These extracts were found to inhibit the growth-promoting effect of thiamin on yeast cells (Endomyces vernalis). The same year SPITZER, COOMBES, ELVEHJEM and WISNICKY demonstrated that chicks in whose diet 25 per cent of raw carp were included developed typical signs of vitamin B_1-deficiency, though the food was otherwise containing adequate quantities of vitamin B_1. Animals fed on raw carp entrails developed polyneuritis earlier than those fed on the heads

and tails or the muscle, meat or skin. Experiments in vitro showed that the inactivation took place in the food-mixture and that it was proportionate to the time of contact with the fish. It was suggested that the action was of enzymatical nature.

In 1943 SEALOCK, LIVERMORE and EVANS reported the results of their continued investigations. They likewise concluded that the rapid destruction by heat, and the precipitability by common protein precipitants indicated that the fish principle was of protein nature. They also considered the factor to be of enzymatical nature and investigated the distribution of the factor in carp viscera. It was present mainly in the majority of the visceral tissues and to a lesser degree in the trimmings (heads, skins, fins and skeleton). On the other hand, the meat ("somatic muscle") contained little or none of the principle. From the results obtained by GREEN and coworkers it could be suspected that the thiamin-inactivating factor might be present in other fishes than carp. In view of the fact that fish is frequently included in the ration of foxes in this country too and that endemic occurrences of Chastek paralysis have also been reported here (CARLSTRÖM and RUBARTH, 1943) it was decided to investigate the distribution of the thiamin inactivating factor in some common Swedish fishes.

Experimental.

Since SEALOCK and coworkers stated that aqueous extract of fresh fish tissues were subjected to a loss of activity even when stored at 5° C, special precautions were taken to exclude the danger of an inactivation of the thiamin destructing factor. The investigation was carried out during the coldest season of the year. Fishes caught in the vicinity of Upsala were bought at the local fish market. Fishes caught in places remote from Upsala were sent to the laboratory as speedily as possible in ice boxes. As soon as the fishes reached the laboratory the entrails of the fishes were dissected free and liver, spleen, pancreas, gastrointestines, gills, kidneys and heart were grinded to a brei and immediately analyzed or stored at —15° C. The figures obtained by SEALOCK and coworkers suggested that testes, ovaries, swim bladder, skin, bone and muscles contained a very low concentration of the factor and therefore they were excluded from the analysis. Before it was discovered that the factor could be stored for months at —15° C without any inactivation occurring, the preparation of a stable dry powder was considered essential. This was accomplished by treating the thoroughly minced viscera with cold acetone or alcohol + ether in the same way as described by SEALOCK and coworkers.

Method of Assay.

Since a chemical method would permit a greater number of determinations in a given period, the colorimetric diazonium method by MELNICK and FIELD (1938) was used. In carrying out the determinations the procedure outlined by SEALOCK and coworkers was mainly followed. To 3 ml aliqouts of fish brei or dilutions of brei, 1 ml of 0.04 M phosphate buffer pH 7.4 was added. Then 1 ml of a thiamin solution containing 700 μg of vitamin was added and after incubation for 2 hours or longer at 40° C, 5 ml of 20 per cent trichloracetic acid solution were added (in the Tables these digestion tests are abreviated to D. T.). The solution precipitated with trichloracetic acid was allowed to stand for 30 minutes in order to obtain a complete precipitation. After centrifugation, 2 ml aliquots of the supernatant fluid were analyzed for thiamin and compared with a solution (in the Tables termed control test = C. T.) which, apart from the fact that the trichloracetic acid had been added immediately following the thiamin addition, and that incubation had been omitted was in every respect identical. The colour obtained in the MELNICK and FIELD reaction was read off by means of a Pulfrich photometer, filter = S 50, 1 cm. cups. The figures obtained were taken to a calibration curve constructed from values obtained with standard solutions of vitamin B_1. A comparison of the figures in Tables 2—4 shows that the absorbtion values of the digestion tests may sometimes be 20 per cent higher than those of the control tests. SEALOCK and coworkers state that an assay of a given extract may be accomplished with an average error not greater than ± 5 per cent. In this work it has been assumed that no real enzymatical inactivation had taken place unless the absorbtion values of the digestion tests were more than 30 per cent lower than the control tests.

Each gram of viscera according to SEALOCK and coworkers yielded on the average 0.15 g of dry powder. In preparing solutions of this material usually 0.5 g of powder was shaken for 1 hour with 10 ml of a 10 per cent sodium chloride solution. The mixture was centrifuged and 3 ml aliquots of the supernatant fluid were analyzed as described above. The different modifications of the method of MELNICK and FIELD usually include a preliminary adsorbtion of vitamin B_1 on zeolite or similar materials. It is stated that this procedure increases the specifity of the method. In the present investigation the adsorbtion technique was not used regularly. The procedure is rather time-consuming and involves further losses of B_1 and — if not absolutely necessary — should be avoided. In several series of analyses a preliminary adsorbtion on frankonit was used. The experimental conditions were the same as given by PLATT and GLOCK (1943), but instead of superfiltrol we used frankonit. The figures obtained with or without adsorbtion of either standard solutions of vitamin or of fish-brei to which vitamin had been added were in complete agreement. Thus the elimination of the preliminary absorbtion of the vitamin was considered permissible in the present investigation. In table 1 the Swedish, English and Latin names of the investigated fishes are given.

Table 1.

The analyzed fishes.

"Asp"	Asp	Aspius rapax (Leske)
Baltic herring	Strömming	Clupea harengus (L.)
"Björkna"	Björkna	Abramis blicca (Bloch)
Bream	Braxen	Abramis brama (L.)
Burbot	Lake	Lota vulgaris (Jen.)
Carp	Karp	Cyprinus carpio (L.)
Char	Röding	Salmo salvelinus (L.)
Cod	Torsk	Gadus callarias (L.)
Crucian	Ruda	Cyprinus carassius (L.)
Gar-fish	Näbbgädda	Belone acus (Risso)
Gurnard	Knot	Trigla gurnardus (L.)
Herring	Sill	Clupea harengus (L.)
Ide	Id	Leuciscus idus (L.)
Lump-fish	Sjurygg	Cyclopterus lumpus (L.)
Mackeret	Makrill	
Melt	Nors	Osmerus eperlanus (L.)
Perch	Abborre	Perca fluviatilis (L.)
Pike	Gädda	Esox lucius (L.)
Pike-perch	Gös	Luciopera sandra (Cuv.)
Plaice	Rödspotta	Pleuronectes platessa (L.)
Roach	Mört	Leuciscus rutilus (L.)
Rudd	Sarv	Leuciscus erythrophtalmus (L.)
Salmon-trout	Öring	Salmo trutta (L.)
Sprat	Skarp Sill	Clupea sprattus (L.)
Tench	Sutare	Tinca vulgaris (Flem.)
Trout	Forell	Salmo irideus (Gibb.)
White-fish	Sik	Coregonus lavaretus (L.)
Whiting	Vitling	Gadus merlangus (L.)
"Vimma"	Vimma	Abramis vimba (L.)
Wolf fish	Havskatt	Anarrchichas lupus (L.)

Results.

The results obtained with extracts on dry viscera from a series of fishes are given in Table 2.

Of all the fishes mentioned in Table 2 only the carp contained a considerable amount of the vitamin inactivating factor. The figures obtained by analysing fresh viscera are given in Table 3.

In the majority of the investigated fishes the vitamin inactivating factor did not seem to be present. It appeared however desirable to secure — if possible — more definite evidence. According to previous investigators, the factor is said to have enzymatical properties and therefore it was assumed that a prolongation of the digestion-time up to 24 hours would increase the difference

Table 2.

The occurrence of a vitamin B_1 inactivating factor in the dried viscera of Swedish fishes.

The values refer to the extinction values given by the diazotized vitamin B_1 content of 2 ml aliquots of trichloracetic acid centrifugates from 3 ml extracts of viscera or other specially mentioned tissues, incubated for 2 hours with 700 μg of vitamin in the digestion tests = D. T. Control tests = C. T. Calculated value of the control test = 0.38. When not otherwise mentioned the viscera were dried with acetone.

Species of fish	Extinction value of		Catched in
	D. T.	C. T.	
Trout	0.37	0.36	Lake at Arjeploug
Trout (extracts of heads)	0.29	0.30	» »
Carp	0	0.22	» Aneboda
Carp (viscera dried with alcohol + ether)	0	0.17	» »
The same extract diluted 1: 10	0.18	0.26	
Carp	0	0.20	Pond at Perstorp
The same extract diluted 1: 10	0.15	0.25	
Baltic herring	0.26	0.28	Baltic Sea (Öregrund)
» »	0.26	0.29	» » »
» »	0.28	0.27	» » »
Burbot	0.28	0.30	Lake Mälaren
Sprat	0.27	0.27	North Sea (Göteborg)
Sprat	0.26	0.27	» » »
Sprat (extracts of heads)	0.25	0.25	» » »
Sprat (extract of muscle)	0.31	0.31	» » »
Sprat	0.29	0.29	» » »
Tench	0.25	0.25	Pond at Dannemora
Tench (extract of heads)	0.29	0.29	» »

between the extinction values of the digestion test and the control test. In Table 4 typical figures resulting from such analysis are given.

The figures in Table 4 show that if the digestion-time is prolonged from 2 to 24 hours a marked difference between digestion test and control test may develop provided the factor is present. This is most clearly demonstrated in the series with the carp brei diluted 1: 20. Significant differences obtained at the end of 2 hours digestion-time with brei from the crucian, ide, gar-fish, tench and vimba increased if the digestion-time was prolonged. On the other hand by extending the digestion time two more fishes, the roach and "björkna" could be added to the series of fishes in which the presence of the thiamin inactivating factor had been demonstrated. Thus the figure of Table 4 are in accordance with those given in Table 3 and support the view that the factor is of enzymatical nature.

Table 3.

The occurrence of a vitamin B_1 inactivating factor in the fresh viscera of some Swedish fishes.

The figures refer to the extinction values given by the diazotized vitamin B_1 content of 2 ml aliquots of trichloracetic acid centrifugates from different dilutions of 3 ml extract of viscera or other specially mentioned tissues, incubated for 2 hours with 700 μg vitamin in the digestion tests = D. T. Control test = C. T. Calculated value of the control test = 0.38.

Family and Species of fish	Extinction value of						Caught in
	Brei		Brei diluted 1: 1		Brei diluted 1: 10		
	D. T.	C. T.	D. T.	C. T.	D. T.	C. T.	
Percidae (Abborrfiskar)							
Perch	0.28	0.24	0.28	0.25			Lake Temnaren
Pike-perch			0.29	0.27	0.30	0.31	» Mälaren
Scombridae (Makrillfiskar)							
Mackerel	0.27	0.33					Kattegat
Cyclopteridae (Sjuryggfiskar)							
Lump-fish	0.30	0.26					»
Triglidae (Knotfiskar)							
Gurnard	0.32	0.28					»
Blenniidae (Slemfiskar)							
Wolf fish	0.31	0.35					»
Pleuronectidae (Flundrefiskar)							
Plaice	0.28	0.30					Baltic Sea
Gadiade (Torskfiskar)							
Burbot			0.20	0.19			Lake Ymsen
Burbot	0.26	0.28					» at Arjeploug
Cod	0.20	0.20					Kattegat
Whiting	0.30	0.31					»
Scomberesocidae (Makrillgäddfiskar)							
Gar-fish	0.12	0.24					»
Salmonidae (Laxfiskar)							
Char			0.23	0.27	0.26	0.25	Lake at Arjeploug
Melt	0.31	0.31					» Mälaren
Salmon-trout			0.27	0.23			» at Arjeploug
Trout	0.37	0.36					» »
White-fish	0.26	0.29	0.31	0.28	0.31	0.29	» »
Esocidae (Gäddfiskar)							
Pike	0.24	0.24					Baltic Sea
Cyprinidae (Karpfiskar)							
"Asp"	0.34	0.34	0.28	0.29			Lake Mälaren
"Bjorkna"	0.26	0.30					» »
Bream	0.16	0.32	0.22	0.33			Baltic Sea
Bream	0.15	0.27	0.18	0.26			Lake Stråten
Carp			0	0.26	0.18	0.30	Pond at Perstorp
Carp			0.09	0.24	0.25	0.31	» »
Carp (extract of muscle)	0.26	0.30					» »

Family and Species of fish	Extinction value of						Caught in
	Brei		Brei diluted 1: 1		Brei diluted 1: 10		
	D. T.	C. T.	D. T.	C. T.	D. T.	C. T.	
Carp			0	0.24			Lake Aneboda
Carp (boiled extract)			0.30	0.29			» Drettingen
Carp	0	0.23					» in Hungary
Carp			0	0.24			» Aneboda
Crucian	0.08	0.25	0.12	0.22			Pond at Dannemora
Crucian	0.08	0.21					Lake Fundbosjön
Ide	0	0.25	0	0.28	0.12	0.28	» Mälaren
Roach	0.25	0.29	0.26	0.29			
Roach	0.30	0.29					
Rudd	0	0.24					
Tench	0.04	0.25			0.20	0.26	Lake Drettingen
Tench	0.10	0.24			0.34	0.27	» Hyltasjön
Tench	0.04	0.24	0.06	0.27	0.20	0.26	Pond at Dannemora
"Vimma"	0.10	0.26	0.12	0.32			Lake Mälaren
Clupidae (Sillfiskar)							
Baltic herring			0.26	0.30	0.30	0.30	Baltic Sea
Herring			0.29	0.28	0.27	0.27	Kattegat
Sprat	0.30	0.28					

The termolability of the factor reported by previous investigators was confirmed. According to the results obtained by SEALOCK and coworkers the optimal temperature of the reaction was 60° C. As demonstrated in Table 5 this temperature can only be used during a very short experimental time (< 2 hours).

In a series of experiments the attempt was made to determine whether the vitamin B_1 inactivation could be correlated with an O_2-consumption. Some of the experiments were carried out in Warburg-Barcroft respirators of common type. The same amounts of viscera extract and vitamin B_1 as previously described i. e. 3 ml brei of carp viscera + 1 ml of buffer solution were used. After 30 minutes 700 μg of B_1 were "kipped" into the brei. In the control tests 1 ml of water was added simultaneously. After a reaction time of 3 hours the O_2-consumption of the control tests and the digestion tests was exactly the same. In another series of experiments the B_1 inactivating reaction was followed with the methylenblue technique in Thunberg tubes filled with nitrogen. The same amounts of carp brei as above i. e. 3 ml + 1 ml buffer solution were used. In both digestion and control tests 0.2 ml of a solution containing 0.8 mg of methylenblue per ml was added followed by 1 ml of vitamin B_1 solution (= 1 mg vitamin) in the

Table 4.

The time course of the thiamin destruction by the fish factor.

The figures refer to the extinction values given by the diazotazed vitamin B_1 content of 2 ml aliquots of trichloracetic acid centrifugates from 3 ml of viscera extract, incubated 2—24 hours with 700 μg thiamin in the digestion test = D. T. Control test = C. T. Calculated value of control tests 0.38.

Species of fish and dilution of viscera extract	Digestion time in hours								Caught in
	2		4		6		24		
	D. T.	C.T.	D.T.	C. T.	D.T.	C.T.	D.T.	C.T.	
Mackerel (brei)	0.27	0.33					0.34	0.33	Kattegat
Lump-fish (brei)	0.30	0.26					0.32	0.26	»
Gurnard (brei)	0.32	0.28					0.27	0.28	»
Wolf fish (brei)	0.35	0.31					0.31	0.31	»
Plaice (brei)	0.28	0.30					0.28	0.33	Baltic Sea
Cod (brei)	0.20	0.20					0.19	0.20	Kattegat
Whiting (brei)	0.31	0.30					0.29	0.30	»
Gar-fish (brei)	0.12	0.24					0	0.24	»
Melt (brei)	0.31	0.31					0.26	0.31	Lake Mälaren
White-fish (brei)	0.26	0.29					0.28	0.29	» Arjeplog
Pike (brei)	0.24	0.24					0.24	0.26	» Mälaren
Asp (brei)	0.34	0.34					0.30	0.32	» »
Björkna (brei)	0.26	0.30					0.03	0.30	» »
Carp (1: 20)	0.32	0.31	0.27	0.31	0.24	0.30	0.14	0.31	» Aneboda
Carp (1: 20)	0.26	0.30					0.14	0.31	» Drettingen
Carp (1: 20) dialyzed 4 hours)	0.27	0.30					0.28	0.31	» »
Crucian (brei)	0.08	0.21					0	0.21	» Fundbosjön
Ide (1: 1)	0	0.28					0	0.28	» Mälaren
Ide (1: 10)	0.12	0.28					0.05	0.28	» »
Roach (brei)	0.31	0.35					0.18	0.35	» »
Rudd (brei)	0	0.24					0	0.24	» »
Vimma (brei)	0.10	0.26					0	0.25	» »
Vimma (1: 1)	0.12	0.28					0	0.30	» »
Tench (1: 1)	0.09	0.29					0	0.29	» Drettingen
Tench (1: 1)	0.06	0.27					0	0.30	Pond at Dannemora
Sprat (brei)	0.30	20.8					0.34	0.29	Kattegat

digestion tests and 1 ml of water in the control tests. This amount of methylenblue was chiefly reduced in 2 hours by the control tests. In several series of experiments it was found that both the digestion tests and the control tests reduced the added methylenblue with the same velocity. Parallel chemical determination demonstrated that in the experimental time chosen (2 hours) the added amount of vitamin was completely destructed. Obviously, the vitamin inactivation is not an oxidation process.

Table 5.

The effect of temperature on the thiamin destruction by the fish factor.

Brei from carp diluted 1: 20, incubated with 700 μg thiamin at 40° and 60° C for 2 respectively 24 hours. The figures refer to the extinction values given by the diazotized thiamin content in 2 ml aliquots of trichloracetic acid centrifugates of brei. D.T. = Digestion tests. Control tests = C.T. Calculated value of the control test 0.38.

Temperature	Digestion time			
	2 hours		24 hours	
	D. T.	C. T.	D. T.	C. T.
40°	0.29	0.32	0.16	0.29
60°	0.29	0.32	0.26	0.28

During the investigation it was observed that in all the fishes, which contained the vitamin inactivating factor the grinded viscera were strongly red coloured. This was only rarely the case with the fishes in which the factor was absent. A spectroscopical examination of a carp viscera extract which was centrifugated for 20 minutes at 15 000 r. p. m. revealed only the presence of hemoglobin. In some experiments a solution of crystallized rat hemoglobin was added to viscera extracts of fishes which did not contain the vitamin inactivating factor. It was however not possible in this way to activate the extracts.

A comparison of the figures of the control tests in Tables 2—4 reveals the fact that only very rarely (the trout in Table 2) is the whole amount of added vitamin regained. From the standard curve it could be calculated that the extinction value of the amount of vitamin present in 2 ml of trichloracetic acid extract (C. T.) should be 0.38. In the different carp extracts the extinction values of the control tests varied between 0.17 and 0.31. This fact is not pointed out by SEALOCK and coworkers. In accordance with these authors we also found the experimental error in the assay of a single fish extract to be about ± 5 %. The low extinction values of the control tests suggests that a reaction similar to that observed in the biotin-avidin relationship may also play a rôle in the inactivation of vitamin B_1 when incubated with fish extracts. It has been demonstrated by EAKIN, SNELL and WILLIAMS, 1940 that biotin in the presence of avidin becomes physiologically nonutilizable through direct combination with the latter rather than through an enzymatic or catalytic reaction

of avidin. An entirely similar situation may exist between thiamin and the fish principle. The low extinction values of the control tests may on the other side result from an unspecific adsorbtion of the vitamin on proteins subsequently precipitated by trichloracetic acid. This question was investigated by adding only so much vitamin as could be calculated to combine completely with some protein in the fish extract in a manner similar to the biotin-avidin reaction. The same amount of fish brei as above i. e. 3 ml + 1 ml buffer were used. Some typical results are given in Table 6.

Table 6.

The adsorbtion of thiamin to trichloracetic acid precipitable substance in the fish extract.

The figures refer to the extinction values given by the diazotized thiamin content of 2 ml aliquots of trichloracetic acid centrifugates from 3 ml of viscera extract.

Species of fish	Added thiamin			
	700 μg		350 μg	
	Calcul.	Found	Calcul.	Found
Cod.....	0.38	0.20	0.19	0.08
Pike.....	0.38	0.24	0.19	0.14

The figures in Table 6 definitely demonstrates that the low extinction values in Table 3 are not caused by some specific fixation of the vitamin to a protein but is only a reflexion of an unspecific adsorbtion to a protein precipitated by trichloracetic acid.

Discussion.

In the present investigation 30 species of fishes were analyzed as to the occurrence of a thiamin destructing factor in their viscera. Of each species several specimens caught in different lakes were investigated. A comparison of the figures of Tables 2—4 demonstrates that the factor was present in ten specimens namely the "björkna", the carp, the crucian, the bream, the garfish, the ide, the roach, the rudd, the tench and the "vimma". Right from the beginning of the reported investigation the attempt was made to elucidate the reason why the factor was present only in certain species of fish. At an early stage when the presence of the factor could only be demonstrated in carps and tenches caught

in the same lake it was assumed that these findings might be explained in this manner that these fresh water fishes, owing to the fact that their haunt of predilection in the lake is there where the water is deepest and the bottom oozy, might be influenced by some factor of metallic or metalloid character present in the bottom layer of certain lakes. A recently published paper by DEUTSCH and HASLER (1943) which in Sweden is unfortunately accessible only in the shape of a brief abstract, supports this view to a certain extent. These authors found that among 31 analyzed species of fresh water fishes coming from the Great Lakes the factor was present in 15; of 9 salt water species none contained the enzyme.

Later on the factor was also found in tenches and crucians which had spent their whole life in cemented ponds permanently filled with water from a well. The factor was also found in breams caught in the Baltic Sea and in the gar-fish, a salt water fish. All these facts did not directly support the view that an external factor might influence the occurrence of the vitamin inactivating factor. At present it can only be stated that the factor was found specially in fishes belonging to the carp family.

The present investigation was not carried out with the intention to analyze the nature of the thiamin inactivating factor or factors occurring in fish. Most investigators are of the opinion that the factor is an enzyme. In accordance with the statements of SEALOCK and coworkers it was found in the present investigation that sometimes a period of considerable length, i. e. of at least 24 hours (Table 4) was necessary until the reaction between the vitamin and the inactivating factor was completed. The temperature optimum found by SEALOCK and coworkers, at 60° C, could not be verified. (Table 5.) The question if there is any connection between the intensity of the red colour of fish viscera and the presence of the vitamin B_1 inactivating factor must be left open for the presence. The results obtained in the WARBURG and THUNBERG experiments do not support the theory that there would be any connection between the factor and some "red" oxidation enzyme.

The reason why the American authors — in spite of the strong positive evidence they succeeded in bringing forth — did not define the factor as an enzyme seems to be the recent disclosure of the avidin-biotin relationship. The interaction between these factors is a rather rapid reaction, whereas the inactivation of thiamin is a more slowly proceeding reaction. Further, as far as

the biotin-avidin complex is concerned, complete recovery of the biotin may be accomplished by heat treatment which alters or destroys the avidin (SNELL, EAKIN and WILLIAMS). When similar heat treatment, proteolytic digestion or acid hydrolysis was employed after incubation of thiamin with the fish principle, SEALOCK and coworkers did not succeed in demonstrating any recovery of the thiamin. Thus, positive evidence supporting the view that inactivation of vitamin B and biotin might occur in a similar way does not seem to be available to date. On the other hand the possibility must be emphasized that both types of reactions might occur parallel. Comparing the figures of the control tests in Tables 2—4 it is obvious that only very rarely the amounts of thiamin added to the controls were recovered quantitatively. Sometimes only 50 per cent of the added amount was recovered. These low yields may be a reflection of a presumably existing mechanism similar to that of the biotin-avidin fixation or only the expression of an unspecific adsorbtion of the vitamin to trichloracetic acid precipitable substances. The latter explanation s supported by the evidence given in Table 6.

Summary.

Among 21 analyzed species of fresh water fishes coming from the Baltic Sea or from Swedish lakes, the presence of a thiamin inactivating factor was demonstrated in 10. Of these 9 belonged to the carp family. Of 9 salt water fishes 1 contained the factor. The character of the thiamin inactivating factor is discussed.

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(From the Biological Institute of the Carlsberg Foundation, Copenhagen.)

Polysaccharide Sulfuric Acids as Anticoagulants.

By

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Recived 5 June 1944.

In continuation of our studies in the chemistry of blood coagulation we have made an investigation on the synthetic polysaccharide sulfuric acids in order to compare their anticoagulant action with the action of heparin.

The anticoagulant properties of synthetic polysaccharide sulfuric acids were discovered by BERGSTRÖM (1935, 1936). He prepared sulfuric acid esters from different polysaccharides by treatment with chlorosulfonic acid and pyridine and found especially the derivatives of chondroitin sulfuric acid, chitin, cellulose, pectinic acid and starch to yield powerful anticoagulants, with the potency ranging from 0.5 to 2 in comparison with 11—13 for pure heparin. Sulfuric acid esters had already been prepared in this manner from starch by TAMBA (1923) and from cellulose by GEBAUER-FÜLNEGG, STEVENS and DINGLER (1928) and TRAUBE, BLASER and LINDEMANN (1932). After the discovery of their anticoagulant properties cellulose sulfuric acid and polyvinyl sulfuric acid have been studied by CHARGAFF, BANCROFT and STANLEY-BROWN (1936) and CHARGAFF and OLSON (1937), while the sulfuric acid ester of starch has been investigated by REUSE (1939). Since the completion of most of our experiments another paper has appeared, KARRER, KOENIG and USTERI (1943), describing the preparation and properties of sulfuric acid esters of certain polysaccharide derivatives. The results of our studies have been published recently (ASTRUP, 1944).

A. Preparation of the Substances.

The various substances have been prepared according to the methods proposed by the above mentioned authors.

Cellulose sulfuric acid: To 30 ml of icecold pyridine 7.5 ml of chlorosulfonic acid are added slowly. 2.5 g of a good grade of cellulose are added and the mixture heated in a boiling water bath for one hour with occasional stirring. After cooling, the mixture is dissolved in 150 ml of water and precipitated with 300 ml of 96 per cent alcohol. After decantation the precipitate is dissolved in 75 ml of water and neutralized with 2-n NaOH. Ten ml of saturated NaCl solution are added and the solution precipitated cautiously with 100 ml of alcohol. After centrifuging, the precipitate is dissolved in 90 ml of water and some insoluble material is removed by centrifuging. The supernatant liquid is precipitated with 90 ml of alcohol, and the precipitate treated with alcohol and dried. Yield 6.1 g. (Preparation No. C-12.) The analyses were carried out after drying to constant weight at 110° in vacuo over P_2O_5. Found: 41.4 per cent ash (as sodium sulfate) corresponding to 13.38 per cent Na; C = 15.39; H = 2.30; S = 18.99. For the trisodium salt of cellulose trisulfuric acid $(C_6H_7O_{14}S_3Na_3)_x$ the following values are calculated: Per cent C = 15.38, H = 1.50, S = 20.53 and Na = 14.75. In accordance with the results of GEBAUER-FÜLNEGG (1928) and TRAUBE (1932) the contents of S and Na lie just under the calculated three atoms per unit of cellulose, in our case S = 2.77 and Na = 2.73, but they correspond to each other and show that the substance may be considered to consist mainly of the trisulfuric acid ester of cellulose. The substance used by CHARGAFF and co-workers (1936) is described as a disulfuric ester.

Pyridine seems to be of special importance to the reaction. Several other tertiary bases (including diethyl aniline and quinoline) were tried without any effect. Technical pyridine bases, which contain no pyridine, gave however a small yield (2.75 g from 2.5 g of cellulose). Different samples[1] made from such bases by fractional distillation were then tried, namely a fraction with boiling point 127—130°, and consisting mainly of α-picoline; a fraction: 142—145° containing β-picoline, γ-picoline and α,α'-lutidine; and a fraction: 156—158° containing α, β-lutidine. Only the lowest boiling of these fractions, the sample containing α-picoline, gave any reaction between cellulose and chlorosulfonic acid, the others were completely inactive in this respect. The reaction did not proceed quite so well with α-picoline as with pyridine, but the yield of cellulose trisulfuric acid was only slightly decreased. Since it is difficult for the time being to obtain pyridine, most of our substances were prepared by means of α-picoline.

It was further found that it was possible to precipitate the cellulose trisulfuric acid by means of concentrated salt solutions, thus by addition of sodium chloride, potassium nitrate, sodium acetate and sodium phosphate (secondary). As a rule our cellulose sulfuric acids were therefore prepared as follows:

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[1] Secured from »Grindstedværket», Grindsted, Denmark.

To 50 ml of α-picoline are added 7.5 ml of chlorosulfonic acid and 2.5 g of cellulose. The mixture is heated $2^1/_2$ hours on a boiling water bath with occasional stirring, and then dissolved in 200 ml of water After neutralization with 100 ml of 2-n NaOH, 100 ml of saturated. sodium chloride solution are added. After decantation, the precipitate is dissolved in 100 ml of water and again precipitated with 100 ml of saturated NaCl solution. After the precipitate is dissolved in 150 ml of water, and centrifuged in order to remove small amounts of insoluble material, the centrifugate is precipitated by addition of 75 ml of 96 per cent alcohol. After centrifuging the precipitate is treated with alcohol and ether and dried at room temperature. Yield 5.6 g (C-31).

Sulfuric acids esters of starch: Only a few samples of these products were made, and all according to the pyridine method described for cellusose. While it was found that rice starch reacted promptly and gave active substances, potato starch reacted more slowly and gave less active products. BERGSTRÖM found rice and potato starch to give almost identical products.

Chitin sulfuric acid: In preparing chitin sulfuric acid in a similar manner, it was found that the substance contained considerable amounts of sodium sulfate as an impurity. While it was easy in the case of cellulose sulfuric acid to remove the salt content by reprecipitations, this was difficult for chitin sulfuric acid, due to its higher solubility in water and salt solutions. Thus it is not precipitated by concentrated solutions of alkali salts, as is the case with the cellulose derivative. The salt content is most conveniently removed by dialysis, but it may also be dealt with by treatment with calcium chloride. While cellulose always yielded very active products the activity of the substances obtained from chitin varied considerably with the conditions of the experiments. Increasing the reaction time decreased the activity and the yield of the products. A shortening of the reaction time and a lowering of the temperature increased the potency of the substances made. The preparation was especially difficult when using α-picoline, and technical pyridine bases yielded only traces of active substance. The most potent products were obtained in the following manner:

To 7.5 ml of chlorosulfonic acid in 50 ml of pyridine (or α-picoline) are added 5 g of chitin and the mixture is heated in a boiling water bath for one hour. After cooling it is dissolved in 200 ml of water and 75—100 ml of 2-n NaOH are added. After precipitating with 500—600 ml of alcohol, the precipitate is dissolved in 200 ml of water and dialysed against running water for two days. After concentration in vacuo to 100 ml, 5—10 ml of saturated NaCl solution are added and the substance precipitated with 150 ml of alcohol. It is treated with alcohol and ether. Yield 4.9 g (K-57). For the analyses it is dissolved in water, dialysed against distilled water, and reprecipitated twice. Found (in per cent): C = 23.7; H = 3.57; N = 3.45; S = 14.4. Calculated for the sodium salt of chitin disulfuric acid $(C_6H_{11}O_{11}NS_2Na_2)x$: C = 23.60; H = 2.72; N = 3.46; S = 15.75. For both cellulose sulfuric acid and chitin sulfuric acid it was found that the activity decreases in the presence of strong acids, yielding inactive substances

of low molecular weight, which pass freely through a cellophane membrane. The products must therefore show neutral reaction if stable preparations are to be obtained. At neutral reaction the solutions may be heated to 100° without decomposition. While during the reaction between chlorosulfuric acid and cellulose practically no cellulose is left unchanged, a considerable amount of the chitin used is left in an insoluble form. Repeating the treatment on the chitin thus isolated from preceding reactions gives only a very poor yield of active substance and most of the chitin has not reacted. The product is probably changed, and is not identical with the original chitin. It may be mentioned that the substance regained from a reaction, unlike the original chitin used, swells in the presence of water.

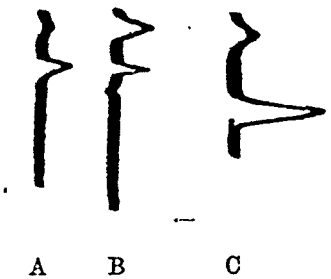

Fig. 1.

A. Cellulose trisulfuric acid (C-12, T-238).
B. Chitin disulfuric acid (K-10) under the same conditions. An anomalous boundary is seen. (T-239).
C. Chitin disulfuric acid prepared by dialysis (K-55, T-224).

Electrophoresis: The substances prepared were all investigated by electrophoresis in order to obtain information about the homogeneity and the relation between contents of components and activity of the products. The electrophoresis apparatus of TISELIUS (1937) and our mirror method (ASTRUP and HELM, 1943) were used. Of the substances 0.1—0.3 per cent solutions in phosphate buffer of p_H 6.7 and ionic strength 0.1 were as a rule used. All the curves showed two components, a fast moving one due to the active substance, and a very slow moving boundary, which was due to impurities of low molecular weight, as it decreased when the substance was dialysed. Some products, which were precipitated from solutions acid to congo paper, showed at the beginning a large amount of the active, fast moving substance, but later it disappeared and only the slow moving substance was left behind. Fig. 1. shows examples of the results obtained.

B. Properties in vitro.

Activity of the Substances: For the approximate determination of the strength of the substances prepared we used the following method, which is not very accurate, but is simple and gives a satisfactory orientation regarding the potency:

5.0 ml of oxalated ox plasma are placed in small tubes and 0.5 ml of the substance dissolved in physiological sodium chloride solution is added. Then the optimal amount of a 1.5 per cent solution of $CaCl_2$, sicc. is added; after turning the tubes, they

are left standing at room temperature. At intervals they are cautiously turned and the clotting observed. The plasma must be as fresh as possible and cannot be kept longer than 3—4 days (at 0°) before it must be discarded. It is necessary to filter the plasma immediately before use. When the clotting is examined, the tubes must not be turned more than once. Shaking the tubes makes the determination uncertain. To control the results a sample of known potency must always be measured at the same time for comparison. As comparison sample we have used a cellulose sulfuric acid ester no. C-4. C-4 is a little less active than the most potent of our cellulose preparations. In Table I an example of such a preliminary measurement of the potency is shown. As stock solutions 0.1 per cent solutions are used. For less potent substances correspondingly larger concentrations must be used.

Table I.

	mg per cent	Hours					
		1/4	1/2	1	2	3	24
Physiol. NaCl	—	(+) (+)	++				
Cellulose trisulfuric acid (C-4)	25	— —	(+) (+)	++			
	50	— —	— —	—	—	(+) (+)	++
	75	— —	— —	—	—	— —	—
	100	— —	—	—	—	— —	—
Chitin disulfuric acid (K-10) (not dialyzed)	25	— —	(+) (+)	++			
	50	— —	— —	++			
	75	— —	— —	++			
	100	— —	— —	++			
Chitin disulfuric acid (K-55) (dialyzed)	25	— —	(+) (+)	++			
	50	— —	— —	++			
	75	— —	— —	—	—	— —	—
	100	— —	— —	—	—	— —	—

It is seen from Table I that the relation between the potencies of K-10 and K-55 is in agreement with the difference between the curves by electrophoresis (Fig. 1). K-55 is about 2—3 times as potent as K-10 and almost as potent as the cellulose sulfuric acid C-4. In this way it is found that the lowest concentrations of the solutions used, with which it is possible to keep the re-

calcified plasma without clots in 24 hours are as follows (in mg per cent of solutions of sodium salts of the sulfuric acid esters): Cellulose: 50; starch: 125; chitin (K-10); 125; chitin (K-55): 75; heparin (about 50 per cent pure): 100. The total concentration of esters in the plasma is only about $^1/_{10}$ of these concentrations. Experiments at the slaughter house with whole blood yield the same results (e. g. 500 ml of blood are kept for several days in a perfectly fluid state by the addition of 30 mg of cellulose sulfuric acid). The potency of the heparin used is about one half of that of the purest heparin preparations. The most active sulfuric acid esters prepared from cellulose and chitin thus show a strength comparable with that of the purest heparin. In this respect our investigations are not in accordance with the experiments published by BERGSTRÖM (l. c.), as his cellulose and chitin derivatives show about one tenth of the strength of pure heparin. It is unlikely that this difference is due to any considerable difference between the constitution of the substances prepared. Rather, the difference may be due to the different measuring methods used, and this possibility is supported by the subsequent investigations. In the experiments of KARRER and *co-workers* (l. c.) the cellulose sulfuric acid is 4 times less active than heparin, while chitin sulfuric acid is only moderately active.

Action of the Substances in vitro: While all previous authors have been interested in the relative strengths of the anticoagulants only, especially in comparison with the activity of heparin, our purpose was to investigate the mode of action of the substances, in order to disclose any differences between the synthetic anticoagulants and heparin.

The equation (1)

$$\frac{1}{t} = k \cdot c^a \text{ or (putting } k = \log b) \qquad (1.)$$
$$\log t = -a \log c - b.$$

was used by FISCHER (1935) to describe the clotting of chicken plasma by means of thrombokinase. Later it was applied to the clotting of oxalated ox plasma with thrombokinase from lung (ASTRUP and DARLING, 1942) and from brain (ASTRUP, 1944) cf. also LEGLER (1943). FISCHER showed that a increased as the heparin content of the plasma was increased, and that for larger concentrations of heparin it was not possible to obtain a straight line corresponding to the logarithmic equation. It was decided to

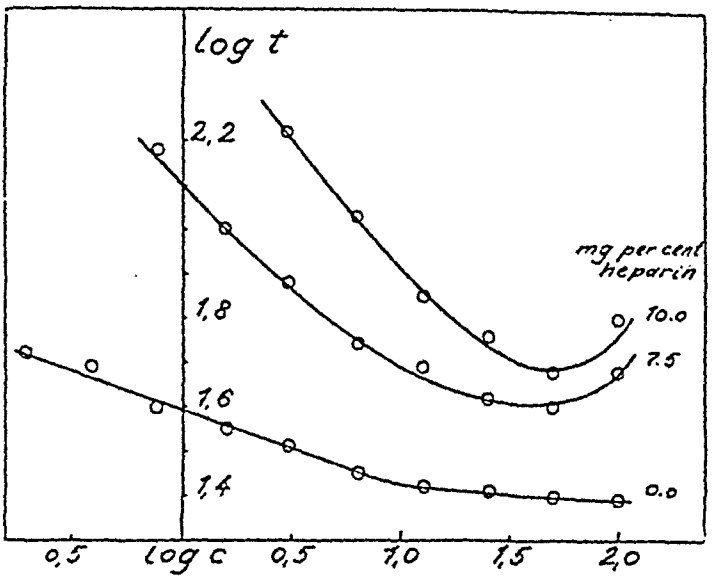

Fig. 2. Clotting of oxalated ox plasma containing heparin, by recalcification and addition of varying amounts of thrombokinase.

study this relation and use it for comparing heparin with the synthetic anticoagulants.

To 45 ml of oxalated ox plasma were added 5 ml of the anticoagulant dissolved in physol. NaCl solution, or in the case of the controls physiol. NaCl alone, and the plasma mixture was used for the determinations as described in the previous papers (l. c.). Some experiments were made with lung thrombokinase, but as a rule brain thrombokinase was used, as it is more stable and may be stored considerably longer without deterioration. The results did not differ in principle, even if it was easier to obtain straight curves with thrombokinase from lung than from brain. Heparin of the strength K = 4—5 was used, cf. ASTRUP and BEHRNTS JENSEN (1938).

Fig. 2. shows that the deviation from straight curves, found by FISCHER by addition of heparin to chicken plasma, also exists in the case of ox plasma. The deviation from straight, parallel lines with a corresponding alteration of the exponent a or an inadequacy of equation (1) indicates changes in the participating reactions, see ASTRUP (1938). It will be seen that heparin is much more effective in preventing the clotting when the amount of thrombokinase is small than when it is large. Presumably this may be due to reversible processes, e. g. a dissociation process in which the action of small amounts of thrombokinase is almost

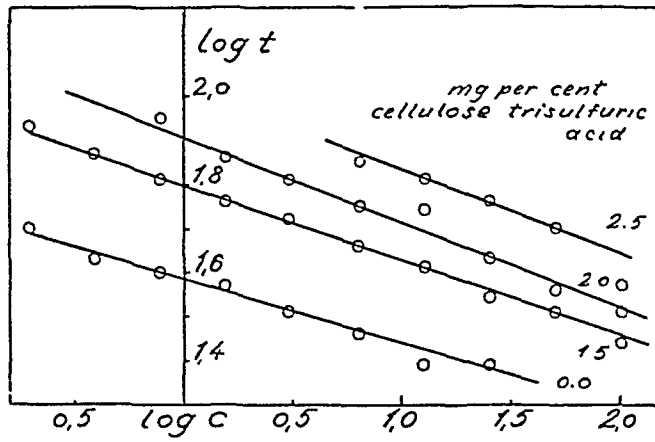

Fig. 3. Clotting of recalcified ox plasma containing cellulose trisulfuric acid.

completely suppressed in the presence of a large surplus of heparin, while larger amounts of thrombokinase are only partly neutralized, thus giving comparatively shorter clotting times.

Similar experiments were carried out with cellulose trisulfuric acid and Fig. 3. shows examples of the curves obtained. The curves are straight parallel lines with the same slope as the original thrombokinase curve. They have the appearance as if various dilutions of thrombokinase had been used as stock solutions (as in the comparison of the strength of kinase solutions). A comparison with the heparin curves in Fig. 2, shows, that the inhibitory action of the cellulose trisulfuric acid is not counteracted by increasing concentrations of thrombokinase to any extent comparable to the effect on the plasma containing heparin. Therefore in the presence of larger amounts of thrombokinase, cellulose trisulfuric acid is a much more powerful anticoagulant than heparin, while at lower concentrations the difference vanishes and at small concentrations heparin is the most potent substance. The findings possibly explain the difference between the activities found by BERGSTRÖM and the activities found by us. It will also be seen from the following curves; that the relative potencies of the anticoagulants in question are completely dependent on the amount of thrombokinase present at the comparison. This is for instance seen in Fig. 4., where heparin is compared with a sulfuric acid from starch and chitin.

Experiments show the sulfuric acid prepared from starch to be similar in action to the cellulose derivative. This is also the case

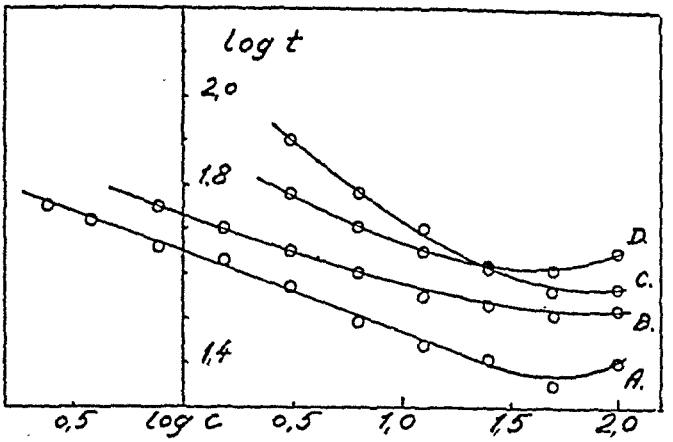

Fig. 4. Clotting of recalcified ox plasma containing: *A:* Physiol. NaCl, *B:* heparin (7.5 mg per cent, k-3.8), *C:* chitin disulfuric acid (15 mg per cent, undialyzed), *D:* starch sulfuric acid (20 mg per cent).

with chitin sulfuric acid at the higher concentrations of thrombokinase, but at lower concentrations deviations are found. The curves tend to deflect at higher clotting times, thus showing a resemblance to the curves obtained with heparin. Hence, the chitin derivative in our experiments seems in the mode of action to lie between heparin and the cellulose and starch polysulfuric acids.

Further experiments show, as is to be expected, that the synthetic polysaccharide sulfuric acids act as antithrombin forming substances in the same manner as heparin. Their action as anticoagulants is therefore similar, qualitatively, to the action of heparin. Thus they prevent the clotting of oxalated plasma by means of thrombin.

The straight lines obtained, e. g. with cellulose sulfuric acid (Fig. 3.), may be explained by assuming that the constant amount of cellulose derivative present inactivates the same fraction of the thrombokinase added, independently of the total amount of kinase. In this manner the new curve will have the appearance as if a new stock solution with a lower concentration of thrombokinase were used. It is however highly improbable, that the inactivating effect of the anticoagulant is independent of the total amount of kinase.

The curves therefore indicate that the reactions (formation of thrombin, action of thrombin) and the inhibition of the reactions do not contribute equally to the proceedings at high and low

concentrations of thrombokinase. Only a chance coincidence between the processes during the reaction may furnish curves like those obtained. Calculations, based on the assumption that the anticoagulant added neutralizes a corresponding amount of the kinase, yields curves, which do not agree either with the curves obtained for heparin or those obtained for cellulose trisulfuric acid.

C. Action in vivo.

According to BERGSTRÖM the cellulose trisulfuric acid is toxic when injected intravenously, and this we have confirmed both in the case of the cellulose and the starch derivative.

Due to the properties of the synthetic anticoagulants, they may in contrast to heparin be useful for technical purposes in obtaining blood and plasma which do not clot. At the slaughter house it is impossible to avoid contamination with relatively large amounts of thrombokinase, and it is well known that under such circumstances heparin, even when used in surplus, tends to yield partial clots in the samples, especially at the surfaces. The greater efficiency of the synthetic anticoagulants in the presence of larger amounts of thrombokinase makes them therefore more suitable for such purposes. As blood and plasma prepared in this manner may serve as a raw material for making foodstuffs, it was found of interest to investigate the toxicity of the substances when given by mouth. It was found by FISCHER and ASTRUP (1939), that heparin when given orally to mice is not absorbed from the intestinal tract but is excreted with the feces, and the synthetic anticoagulants were therefore expected to behave similarly and to be nontoxic to the organism. Our experiments showed this to be true. Thus mice were given, by mouth, daily in 10 days 50 mg of the cellulose trisulfuric ester, without showing any sign, macroscopically or microscopically, of toxicity.

The chitin disulfuric acid was also tried and was found in our experiments to be nontoxic also when given intravenously. While rabbits, when given 100 mg of cellulose trisulfuric acid dissolved in 2 ml of physiol NaCl solution died in the course of about two hours, we gave 200 mg of a chitin disulfuric acid to rabbits daily in five days intravenously without toxic phenomena. The chitin derivative was thus found to be far less toxic than the cellulose and starch derivative, and more like heparin in this respect. It may be mentioned that the polysaccharide skeleton of heparin

is more like that of chitin than it is like that of cellulose or starch; heparin, as far as is known consisting of acetyl glucosamine and hexuronic acid residues, chitin of acetyl glucosamine residues and cellulose and starch of glucose residues.

Owing to the considerable interest in an inexpensive substitute for heparin, we have turned our attention to this problem. The whole question of the pharmacology of synthetic polysaccharide sulfuric acids has been investigated by PIPER, and some of his results will be published shortly. Using a new method for the determination of the toxicity, based on the discovery that the blood platelets are agglutinated in the presence of the substances considered, PIPER finds that chitin disulfuric acid is not quite so harmless to the living organism as heparin. The substance also agglutinates the platelets, although as a rule not to the same extent as cellulose and starch polysulfuric acids. This occasionally results in toxic phenomena in the animals (infarcts), but only single cases of death are encountered, and that only when using very large amounts of the substance. However, chitin disulfuric acid, too, must be considered too toxic for use in medicine, but may serve as a useful substitute for heparin in experiments on animals. It is used in about the same doses as heparin ("Leo" or "Vitrum") and such amounts may be considered completely harmless to the animal. The substances prepared by KARRER (l. c.) are found in recent experiments also to agglutinate the blood platelets, thus being more or less toxic.

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Summary.

1. The preparation of synthetic polysaccharide sulfuric acids is studied, and their properties as anticoagulants investigated.

2. A convenient method for the approximate determination of the strength of the anticoagulants is described.

3. It is found that the action of the synthetic derivatives differs from that of heparin in that it is less influenced by large amounts of thrombokinase.

4. While they are quite harmless when given orally, they are more or less toxic when injected intravenously. Cellulose and starch

sulfuric acids are the most toxic of the substances investigated, while chitin disulfuric acid is only slightly toxic. The last mentioned substance may be used as a suitable, inexpensive substitute for heparin in experimental work.

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From the Laboratory of the Medical Clinic Lund.

On the Presence of a laccase-like Enzyme in Serum and its Relation to the Copper in Serum.

By

CARL G. HOLMBERG.

Received 7 June 1944.

On a visit to Upsala in 1942 my attention was drawn by Dr K. O. PEDERSEN of the Physico-Chemical Institute there to a strongly blue-coloured globulin, now and then found in swine serum.

When fractionating euglobulin according to GREEN (1938) I observed that the P_I-fraction was occasionally markedly blue-coloured. After the attention has once been drawn to this fact, it is not hard to see that this fraction always has a more or less marked blue tinge.

It has not been possible to work out which factors determine how strong the colouring of the P_I-fraction shall be in any one case. We have observed the strongest blue coloration in some cases of polycytaemia rubra vera. Other cases of this disease, however, have only shown a very faint blue coloration of the P_I-fraction.

With a strongly coloured P_I-fraction, it is easy to show that the colour disappears with the addition of a reducing agent and returns, if the solution is bubbled through with oxygen. No distinct absorption band can be demonstrated in the visible spectrum, even with the most strongly coloured solutions.

The Copper Content of the P_I-fraction.

As the colour recalls the blue tint in the oxidation enzyme laccase, isolated by KEILIN and MANN (1938) it was of a certain

interest to investigate whether, like the laccase, this globulin fraction also contains copper. This was found to be the case. About 8—9 % of the copper content of serum was found in the P_I-fraction. As this fraction only represents a very small part of the entire serum protein, we have here an accumulation of copper. That is to say, this protein fraction contains proportionally about five times so much copper as the serum protein as a whole. The combined P_{II}—P_{III}-fraction, obtained with GREEN's method, contains, on the other hand, if anything less copper than the total protein.

In the physiological increase of the copper content of serum during pregnancy, the amount of copper in P_I and the total serum copper increases proportionally.

The highest copper content as yet found in a P_I-fraction is 0,23 ‰. The blood sample was from a pregnant woman, and the serum copper had been determined at 268 γ per 100 cc.

The Enzymatic Properties of the P_I-fraction.

As the blue colour could be reversibly oxidized and reduced, it was natural to suspect the presence of an oxidation catalyzer in the P_I-fraction. This has therefore been tested in Warburg experiments in the presence of the following substances: paraphenylendiamine, paracresol, catechol, adrenalin, ascorbic acid, pyrogallol and thyrosine. Only the first of these substances was oxidized in the presence of the P_I-fraction.

The rapidity of the oxidation showed good parallelism with the copper content. A quantitative assessment was however difficult, as P_I-fractions poor in copper showed a more or less marked period of latency, while fractions rich in copper showed a constant oxygen-consumption. In the P_I-fraction, which was richest in copper, Q_{O_2} was about 10 at 37° C and in air.

The oxidation of paraphenylendiamine was completely inhibited by 1/1,000 molar KCN.

The addition of a surplus of cytochrome C did not accelerate the oxidation of paraphenylendiamine.

If Cu in the form of copper sulphate was added to a P_I-fraction poor in copper, the latter's activity was increased to about that found in fractions rich in copper.

All the Warburg experiments were performed at pH 7.5 in a

phosphate buffer. In the experiments with added copper the controls received the same amount of copper as in the enzyme experiments.

Summary.

The assumption of EISLER, ROSDAHL and THEORELL (1936) that all serum copper is bound to the albumin does not prove quite true. A small amount of the total serum copper is to be found in the euglobulin fraction, which GREEN has called P_I.

KEILIN and MANN's suggestion that the haemocuprein is enzymatically inactive does not refer to all the protein-bound copper in the blood. In GREEN's P_I-globulin there is a fraction which catalyzes the oxidation of paraphenylendiamine. The proteine component necessary for the formation of this enzyme seems normally to be present in surplus, so that the addition of copper is able to increase the activity.

The blue colour of the active globulin fraction has no importance for its catalytic function. It may possibly be due to some oxidation product with great affinity for the enzyme.

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From the Laboratory for the Theory of Gymnastics,
University of Copenhagen.

Temperature Measurements in Human Muscles in Situ at Rest and during Muscular Work.

By

FRITZ BUCHTHAL, POUL HØNCKE and J. LINDHARD.

Received 26 June 1944.

In connection with earlier investigations dealing with the physical and chemical processes which occur in the skeletal muscles during contraction and restitution we have carried out temperature measurements in human muscles, partly while the muscles were at rest and partly during and after work or static muscular activity. Simultaneously, or at least at short intervals, the rectal temperature has been measured during the experiments in order to obtain a basis for evaluation of the muscle temperatures. For the purpose of deciding, if possible, whether any rise in temperature that might occur in the muscles is due to an increase of the circulation, or is caused by processes with positive heat effects in the muscles themselves, we have moreover conducted experiments during which the circulation of the extremities concerned was arrested.

Method.

The temperature measurements were carried out electrically by means of needle-shaped thermocouples in the muscles. The thermoneedles used were 18 mm long and 0.5 mm thick; they were equipped with a celluloid collar which ensured that the needle always was inserted to the same depth. The thermocouple consisted of a hollow needle of stainless steel (V2a) with its tip cut off at a slant. A double-covered constantan wire, 0.1 mm thick, was inserted into the hollow

of the needle and soldered to the tip (fig. 1). The "cold" junction was placed in a Dewar flask (1) at a temperature of about 30°. The recording instrument was a Hartmann and Braun light spot galvanometer, which for the sensitivity employed had an inner resistance of 0.047 ohm, while each division on the scale corresponded to 22.60×10^{-6} amperes.

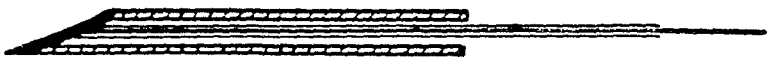

Fig. 1. See the text.

The assembly (fig. 2 a) was calibrated by placing the thermo-needle in another Dewar flask (2) together with a mercury thermometer, care being taken to have the thermocouple and the Hg-thermometer at the same level and close to each other in the Dewar flask. The temperatures in the Dewar flasks were read with an accuracy of 0.05°. The temperature in Dewar flask (1) was set at different levels and for each a number of galvanometer readings were obtained, corresponding to different temperatures in Dewar flask (2). These experiments, which were carried out with each one of the thermo-needles used, showed a completely rectilinear dependence as regards the temperature difference and the galvanometer readings. The location of the line was almost the same for the different thermo-needles. On the average, 6.5 divisions on the galvanometer corresponded to 1°. Since there was no difficulty in judging one-tenth of the intervals it was possible to read off the temperature with an accuracy of approximately 0.015°. This set-up was well suited for the study of all the temperature fluctuations that

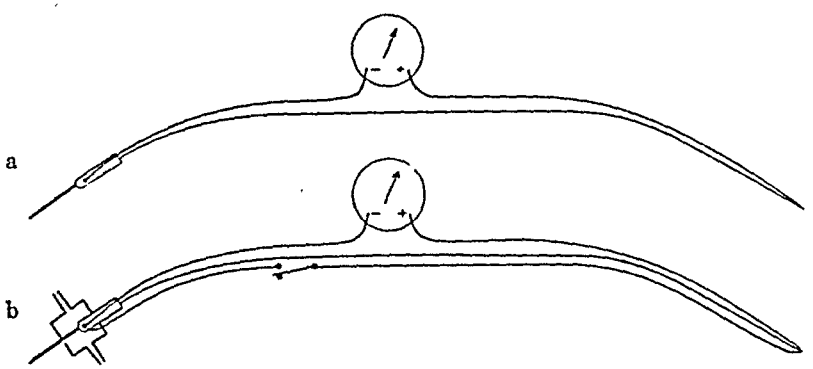

Fig. 2. Assembly for measurement (a). Control arrangement (b). For explanation, see text.

occurred. Since it was anticipated, however, that heat might be lost from the upper part of the thermo-needle, in spite of the celluloid sheath, giving rise to a systematic error on the absolute temperature determinations, the following precautions were taken: On to the upper part of the steel tubule there was soldered a constantan wire of the same thickness as the one soldered to the tip. It was con-

nected to the wire leading to the Dewar flask (1) through a switch (entirely of constantan) in such a way that it was possible, easily and rapidly, to shift between the wires from the two junctures (fig. 2 b). The upper part of the thermocouple was then surrounded by a tube which was filled with water from a mixing reservoir; the temperature of the water was varied until the galvanometer no more showed any deflection when reversing the constantan switch. *i. e.*, until the two junctions had the same temperature. The results of these experiments showed that at the room temperature, maintained at about 22°, there was a systematic error of the muscle temperature of very close to — 0.5°. A correction for this error has not been applied to the material, since it was the temperature fluctuations we were interested in. When in the following absolute values for the muscle temperature are given, they should, therefore, when nothing else is said, be increased by 0.5°. BAZETT and MCGLONE (1927) report that in temperature measurements on the upper extremity they occasionally find lower temperature in the depth than at the surface of the skin, and they consider this to prove that the needle-electrode is encumbered with a systematic error, an error for which they try to allow by means of a special logarithmic formula. There are several reasons for viewing this whole idea with some scepticism. One reason is that it is hardly justified to accept the authors measurements of the skin temperature as reliable — another, that measurements of this kind, as shown for example by our experiments, involve so many different sources of uncertainty that a correction by means of a complicated formula is not very reassuring. If a correction is required there is no doubt that a purely empirical one is preferable.

When 2 thermo-needles were placed in the same muscle, or needles were placed simultaneously in symmetrical muscles, the wires were run via a switch to the galvanometer in such a way that merely by turning the switch it was possible to connect the desired thermo-needle to the circuit. Since the galvanometer needed only a few seconds to adjust itself it was possible to make the two measurements in such rapid succession that they might be considered simultaneous.

The rectal temperature was measured by means of a thermocouple of the type previously described by HOHWÜ CHRISTENSEN (1931). The junction was covered with sealing wax, and from there the wires were protected by a thin rubber tubing. The junction was moved 10—15 cm up into the rectum where it remained for the whole experimental period. This thermocouple was calibrated in the same way as the thermo-needles. In a few instances the rectal temperature was measured only once at the beginning or at the completion of the experiment by means of an ordinary Hg-thermometer.

During the experiments the subject was placed in a sitting position on a low chair; his arms and the upper part of his body were exposed, but, as a rule, he had a blanket over his shoulders. During the work experiments the upper arm rested, with rectangular flexion in the shoulder, on a horizontal, slightly upholstered support. To ensure the uniformity of the position and facilitate the orientation, both

the upper arm and the forearm were surrounded by 2 upholstered metal splints, hinged together, and in connection with a graduated arc. The arm remained in the same position during the whole restitutional period, or, at any rate, as long as the experiment lasted. Since the time that lapsed before the muscle temperature became relatively constant was rather long — most frequently about 12 minutes — an attempt was made to shorten it. To this end the upper arm was, in some experiments, partly surrounded by an electrically heated pad, or loosely wrapped in flannel. However, inasmuch as such measures did not demonstrably influence the results they were again abandoned. Dynamic work consisted in raising a weight in a cord running over a pulley to a ring in a leather cuff or belt, 3 cm wide, fitted to the wrist of the subject immediately above the hand. The flexion angle in the elbow in the initial position, and the flexion interval through which the raising of the weight took place, were recorded so that the experiments at all times could be reproduced. In experiments with static muscular activity (in the following called static work) the position of the subject was the same. The flexion angle in the elbow was controlled. The dynamic work was done in time with a metronome which, as a rule, was adjusted to 34 oscillations per minute. The work time varied with the nature of the work and the load, as well as with the strength and condition of the subjects. In the experiments where the circulation was arrested, the arresting was done by means of a tourniquet placed as high up on the arm as possible. With one exception, the working muscle group was the flexor muscles of the elbow. In most experiments 2 thermo-needles were stuck into m. biceps, one a little above the most prominent part of the muscle and the other distally at a distance of about the width of 3 fingers. In the rest experiments and towards the end of the restitutional period the galvanometer readings for the 2 needles were made immediately one after the other, so that the two readings could be said to represent the same po nt of time. In all work experiments the galvanometer readings were made alternatingly at intervals of 15 seconds. and the same applies to the beginning of the restitutional period. After a few minutes the intervals were gradually increased to 2—3 minutes.

It frequently happened that the two needles in the same muscle did not give corresponding results as far as the absolute temperature was concerned; the curve representing the variation was, however, the same. In nearly all instances the distal needle showed a lower temperature than the proximal one. There may be several reasons for this phenomenon. One is that the temperature in the extremity as a whole is falling distally — another that the temperature is falling from the depth to the surface (cf. GRANT and BRUCE PEARSON (1937). We too have found that the results varied with the depth to which the needle was introduced; moreover, that the sliding-out of the needle manifested itself as a fall in temperature, just as it gave too low results if a needle was stuck in at an angle instead of perpendicular to the surface. The whole problem seems, however, to be even more involved than that. Thus we have found noticeable temperature differences with needles

that were placed only 1—2 cm apart. However, this lack of agreement between measuring results from the same muscle at the same time is hardly due to technical errors alone; it is probable that it is mainly connected with the structure and the innervation conditions of the muscle. Since the number of motor ganglionic cells is far smaller than the number of muscle fibres, each ganglionic cell will innervate a certain, usually quite large number of muscle fibres which thus come to form a mechanical unit ("motor unit", SHERRINGTON). A motor unit is no histological unit, however, since the individual fibres within this unit may be placed at some distance apart within the muscle bundle. Since, moreover, the contraction of the muscle in our experiments is not maximal, all motor units are not simultaneously engaged — and since,

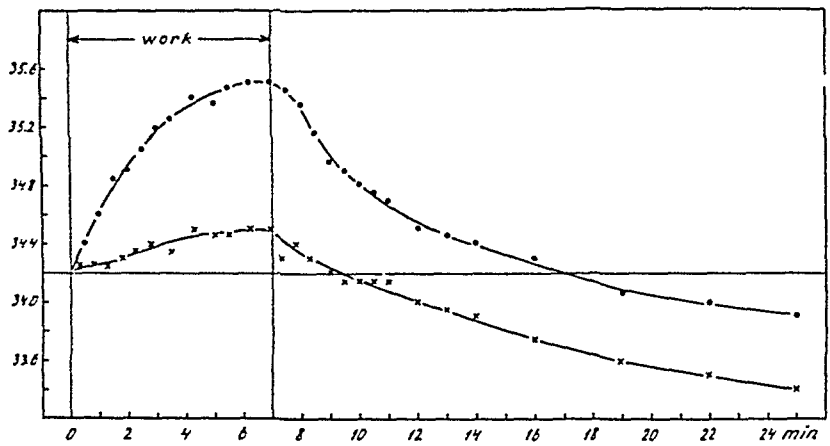

Fig. 3. P. H. Load 2.5 kg. Upper curve from the proximal, lower from the distal thermo-needle.

finally, the slender thermo-needles have a relatively limited "radius of action", it will be understood that it is quite possible that irregularities like those mentioned may occur. What is said here is especially true when the load on the muscle is small. A very glaring example is recorded in fig. 3. Subject P. H., load 2.5 kg. The upper curve originates from the proximal, the lower one from the distal needle There can be no doubt that the distal needle in this case has not been in the immediate proximity of active muscle elements (compare fig. 9). Lastly, it is possible that a needle may pierce a large blood vessel or be placed close to such a vessel. As a rule, however, these different irregularities are not too large to be smoothed out by average curves from a few experiments.

Muscles at Rest.

The temperature in the resting extremity muscles is considerably lower than the rectal temperature, as already demonstrated by several investigators. Thus GRANT and BRUCE PEARSON (1937—38)

find, in the dorsal muscle group of the forearm, at a depth of 2 cm, a temperature of 34.1°, which in 75 minutes falls to 29.4°. BAZETT and MCGLONE (1927) find that the muscle temperature of the forearm "close to the bone" is 1—2° lower than the rectal temperature. Our experiments give similar results. BARCROFT and MILLEN (1939) report the temperature in the gastrocnemius to be 37°, which must be considered a very peculiar result. In order to cool the muscles these authors place the extremity in a water bath at 32.5°, which in the course of 2—3 hours results in a temperature in the gastrocnemius of 34.5°. From what is said above it must be assumed, however, that the water bath has *prevented* a temperature drop in the extremity, the temperature of which, after 2—3 hours at complete rest in air, undoubtedly

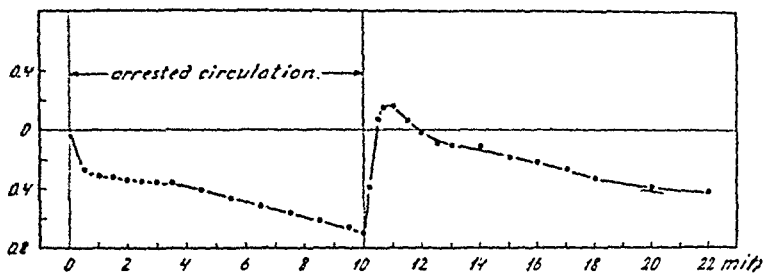

Fig. 4. P. H. Rest. Circulation arrested. Zero-line = 34.7°.

would have been considerably lower. The temperature level varied with the individual, just as it may vary from day to day for the same subject. The exposure of the arms and the upper part of the body resulted, as a rule, in a sharp drop of the temperature in the muscle; in 10—12 minutes this changed to a slow fall which was continued during the whole time of the experiment. Arresting the circulation to the arm, after the slow temperature fall had begun, again led to a temperature drop in the biceps of 0.3° in the first $^1/_2$ minute, whereupon the fall slowed down. In one case (fig. 4) the temperature fell 0.7° in the course of 10 minutes: then, when the circulation was restored, it rose in 1 minute to about 0.2° above the initial value, thereupon falling again. After 10 minutes the temperature was 0.4° below the initial value.

As already mentioned, the majority of our experiments deal with the biceps brachii. It was found, however, that the temperature in different muscles differed considerably (though the differences were only small in symmetrical muscles). For the

purpose of giving a picture of the magnitude of these differences, temperature measurements were made in a number of muscles, partly on the truncus, partly on the extremities, of 2 different subjects. The results were, apart from an individually different amplitude of variation, very uniform. Fig. 5 records the mean values from 2 experimental series with F. B. Inasmuch as thermo-needles were placed simultaneously in symmetrical muscles, and since 2 readings were taken for each muscle, it follows that each point of the curve is the mean value of 4 measurements. As fig. 5 shows, the highest temperatures in the muscles investigated by us were found in the two shoulder muscles, infraspinatus and deltoideus (II) where the temperature was very close to the rectal temperature; the temperature in the pectoralis major was only 1° while in the sacro-spinalis lumbalis it was 1.5° below the rectal temperature. The temperature in the muscles of the extremities is, however, much lower than in the body muscles, and falls distally, with a considerable gradient. In the case of the upper extremities we find a decreasing temperature in the following sequence: biceps, triceps, brachio-radialis (on the forearm); in the latter, the temperature is 2.5° lower than the rectal temperature. Then follows a considerable fall, the temperature in the thenar being not less than 4.5° lower than the rectal temperature. A corresponding temperature fall is found in the lower extremities, where the temperature has been measured in the rectus femoris, the tibialis anticus and the gastrocnemius lateralis. In the last mentioned muscle the temperature is about 3° lower than in the shoulder muscles. The temperature in the muscles of the feet has not been measured. During the measurements, the muscles have in all instances been relaxed, and none of them have immediately before been in action to any degree worth mentioning.

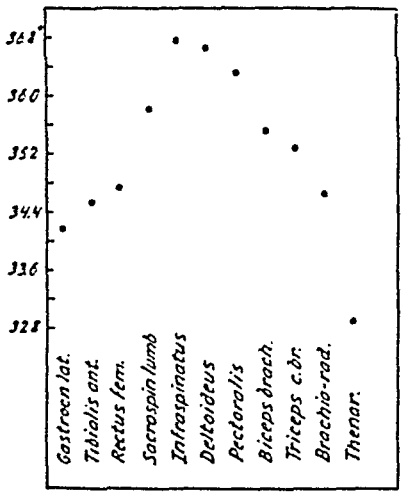

Fig. 5. F. B. Temperature in different muscles under the same experimental conditions. Each point the mean of 4 measurements.

It is impossible to say for certain what is the cause of these temperature differences The factors which must be considered

in this connection are the heat production in the resting muscles, the heat supplied by the blood, and the heat loss.

It can be established immediately that the low temperatures, of course, are not something which is characteristic for the musculature as such; the temperature of the extremity as a whole lies lower than the rectal temperature, and it decreases strongly in the distal direction. The heat production that occurs in the upper extremity is practically confined to the musculature; when the muscles are at rest it is not considerable. According to an estimate by ASMUSSEN, HOHWÜ CHRISTENSEN and NIELSEN (1938), based on metabolic experiments with arresting of the circulation to the lower extremities, 20—25 per cent of the organism's metabolism at rest should take place in the musculature. However, it is only a small part of the musculature (about 5 per cent) that belongs to the upper extremity in its narrower sense, and one may therefore confidently assume that the production of heat here is very small when the muscles are resting. It probably remains a fact, though, that the heat production in the muscles must be considered the most important source of heat in the extremity; the other source of heat, the blood that circulates in the extremities, must come second. BAZETT and MCGLONE (1927) have tried to measure the temperature in the blood vessels themselves, and even although these investigations are rather fragmentary, they should not be overlooked. The authors found in the large and well protected arteria femoralis temperatures which were about 1° below the rectal temperature. On the upper extremity they found arterial temperatures in the plica cubiti of 35—36°. The subcutaneous veins could be ignored, since they, as a rule, were strongly contracted; in the deeper lying venae comitantes the temperature was found to be considerably lower than in the arteries, and the authors reached the conclusion that the close proximity to these veins was one of the reasons for the relatively low arterial temperature. It is quite evident, though, that another reason for the low blood temperature is the slowness of the blood stream. One may estimate the average rate of flow of the blood in aorta, at rest, to be 11—12 cm/sec., and this velocity becomes less and less as the arteries branch out. In the small arteries to the arm muscles the rate of flow must therefore be quite low. Arresting the circulation to the upper extremity (fig. 4) results in a sudden, but small fall in the muscle temperature, and the fall continues, but more slowly, as

long as the circulation is shut off. It seems reasonable to conclude that the first fall of the temperature curve is due to the cutting off of the heat supplied by the blood, while the subsequent decrease is due to an arresting of the oxidation processes in the muscle.

When GRANT and BRUCE PEARSON (1937—38) are of the opinion that the low temperature in the muscles *proves* that the circulation in the resting muscles is very small, then we must consider this, at its best, a less fortunate expression, since it does not explain the considerable fall in temperature in the distal direction. It is very improbable that vascularisation and the flow of blood should be greater per unit of tissue in the large, coarse shoulder muscles than in the much used and finely co-ordinated small muscles of the hand.

There is nothing strange, however, in the circumstance that the rectal temperature can remain relatively high. A considerable heat production takes place in the trunk, partly due to the activity of the large glands and partly due to muscular activity. The muscles concerned are the heart and perhaps the smooth musculature of the intestines, the respiratory muscles and the muscles that participate in maintaining the active positions of the body.

If we now consider the second main factor, the heat loss, the situation is quite similar. The surface of the trunk is small in proportion to the volume, while the surface of the extremities, especially of the upper extremities, is relatively large. Moreover, the heat insulating layer, the subcutaneous layer, is more strongly developed in the trunk than in the extremities, and in the latter more pronounced in the proximal than in the distal part. On the other hand, it must be remembered, bearing our experiments in mind, that a more developed subcutaneous layer with the method used by us must give cause for lower temperatures since the thermo-needles are introduced to the same depth. Everything else being equal, this should cause higher measuring results in the thenar than in the pectoralis, something, though, which apparently is of secondary importance. It is the heat loss that dominates the picture. This heat loss also explains the large temperature drop from the brachio-radialis to the thenar, the lower third of the antibrachium having very little musculature and a large surface the shape of which favours the heat loss; thus no heat is produced and the arteries to the hand lie close to the surface without muscle covering.

Taking all this into account one must be justified in assuming that the low muscle temperature in the upper extremity is due to a small heat production in connection with a high heat loss, while the quantity of heat supplied by the blood appears to be of minor importance.

Experiments during and after Muscular Work.

The work experiments, like the experiments at rest, did not start until the temperature in the resting muscle had reached a relatively constant level, *i. e.*, when 2 or 3 measurements at intervals of 1 minute gave practically the same result. This initial value varied from one individual to the other, and for the same subject from day to day. Inasmuch as this circumstance often made it impossible to make a direct comparison between the experimental results, we preferred as a rule to consider the initial value as 0-value, and, when drawing the curves, to record rise or fall relative to this value. In this way it became possible to express the results of several experiments in an average curve. However, the mean curves mentioned in the following represent the experiments with one subject only, since the individual variations did not appear in the initial temperatures alone. In the beginning of the investigation we frequently undertook several experiments on the same subject on the same day. It was soon discovered, however, that one could not compare the 1st and the 2nd experiment, *inter alia* because the latter always gave a smaller, frequently considerably smaller, temperature reaction than the former. The program was therefore changed to include only 1 experiment on the same person on a single day. As an exception, however, experiments were made on the same day with symmetrical muscles in the same person. The experiments involved partly dynamic and partly static work, and within each of these groups both the experimental time and the load were varied. Finally, experiments were made in which the circulation was arrested. The subjects were all healthy men in the age from 25 to 40.

Fig. 6 shows the results of 3 experiments on P. H. made on the same day; time between the experiments about 1 hour, dynamic work, the load (the same in all 3 experiments) 5 kg. The experiment was each time continued until fatigue set in. In the 1st experiment the work was interrupted after 4.5 minutes, in

the 2nd after 4, and in the 3rd after 3.5 minutes. When the temperature curve, as in these instances, begins to fall before the work ceases, it must be considered a pronounced symptom of fatigue. In the I-experiment the temperature rise is 1.9°, in the

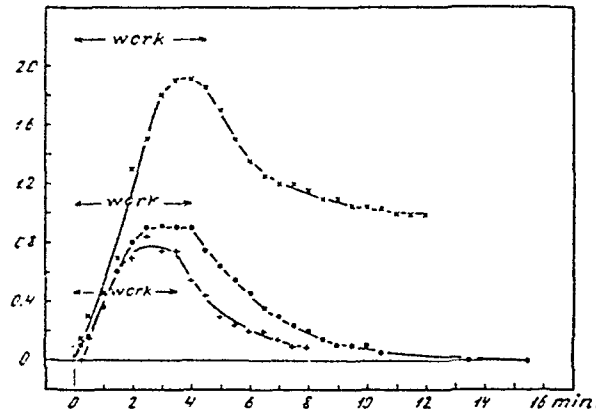

Fig. 6. P. H. Dynamic work. Load 5 kg. 3 experiments at intervals of about 1 hour. Zero-line resp. 36.2, 37.1 and 37.1°.

II-experiment only 0.9°, and in the III-experiment not quite 0.8°. The restitutional period shows similar conditions. In the 1st experiment the muscle temperature is still 1° above the initial value after 8 minutes, while in the other experiments it rapidly reaches the 0-line.

Fig. 7 shows likewise the effect of preceding work. F. B. dynamic work, load 5 kg. Each curve represents 3 experiments, so that a point is the mean value of 6 measurements. The work

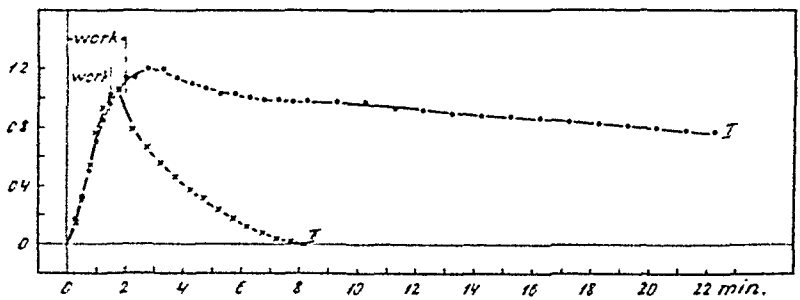

Fig. 7. F. B. Dynamic work. Load 5 kg. Upper curve: I-experiment, lower curve II-experiment. Zero-line I = 35.1, II = 36.3°.

time is 2 and 1.5 minutes respectively. The temperature rise continues after the work has ceased. The individual difference between this and the foregoing experiment is especially seen in the restitutional period, which in the I-experiments is very pro-

tracted: after 20 minutes the muscle temperature is still 0.8° above the initial value. In return, the temperature drop in the II-experiments is exceptionally steep, something that seems to be characteristic of the subject in question; we see it again in fig. 7 a which likewise is a II-experiment. Here the load is 10 kg, the experimental time shorter, and the temperature fall in the restitutional period still more precipitous. — In spite of the differences pointed out, the temperature reaction is nevertheless in principle the same in both subjects.

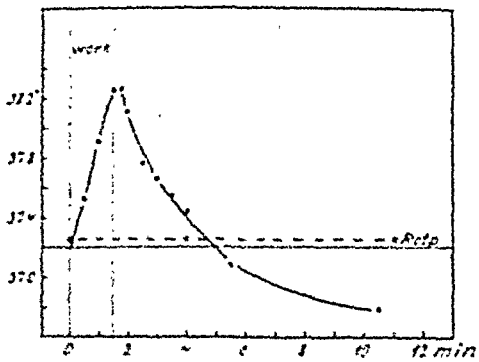

Fig. 7 a. F. B. Dynamic work. Load 10 kg. II-experiment.

The cause of the above mentioned difference between the 1st and the 2nd experiment was undoubtedly that the interval between the two experiments was too short, 1—1.5 hours so that the fatigue caused by experiment I had not disappeared when experiment II was started. Practical reasons made it impossible however, to extend the experiments over a longer period of time, so that it became necessary to be satisfied with 1 experiment per day for each subject.

Dynamic Work.

Fig. 8 records an experiment in which the work was done by the dorsal flexors of the foot, with the thermo-needles in the tibialis anticus, both in the active muscle and in the symmetrical resting muscle. During the experiment the foot rested on the heel, and the work consisted in raising a weight of 5 kg attached to the forepart of the foot. The temperature in the working muscle shows after 0.5 minutes a slight fall, then rising rapidly and strongly until it is 2.5° above the initial value

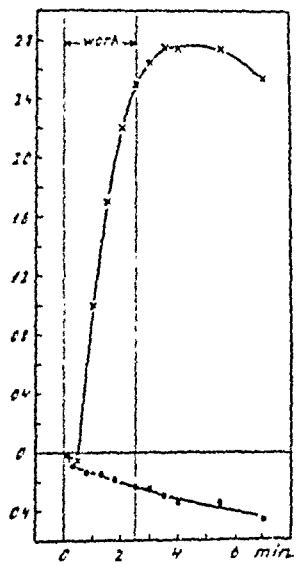

Fig. 8. F. B. Dynamic work with the dorsal flexors of the foot. Load 5 kg. Upper curve from the working tibialis anticus, lower curve from the symmetrical resting muscle.

after 2.5 minutes, when the work stops. In the restitutional period the temperature shows a further small rise, but begins to fall after a couple of minutes. For some reason or other the experiment has been stopped too soon. In the resting muscle the temperature falls throughout the entire experiment, in the course of 7.5 minutes 0.5°.

Fig. 9 shows an average curve resulting from 2 work experiments, one with the right and one with the left arm. Simultaneous measurements have been made of the temperature in the sym-

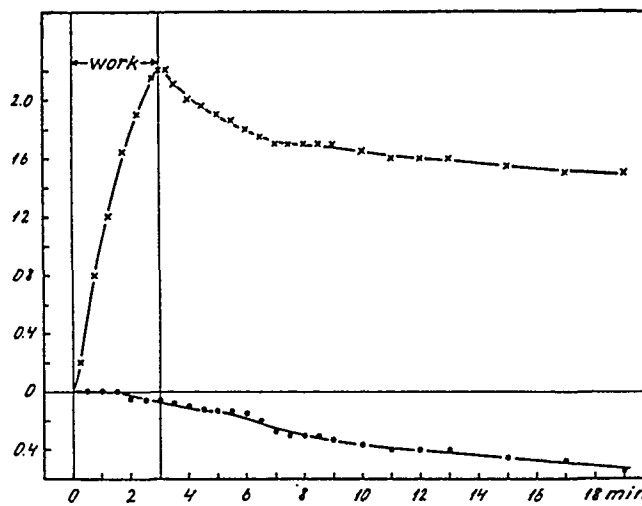

Fig. 9. P. H. Upper curve giving mean values from 2 work experiments, one with the right and one with the left arm. Load 5 kg. The lower curve shows the temperatures in symmetric muscles in the resting arm.

metrical muscle. Subject P. H., load 5 kg. The temperature in the muscle rises 2.2° during the work. After the work is over the rise changes promptly to a fall; after 16 minutes the temperature is still 1.5° above the initial value. In the resting arm the temperature is steadily falling during the experimental period. The temperature fall in the resting extremities does not show any demonstrable difference from what is found when both extremities are at rest.

The temperature increase in a muscle during work *increases with the load*, when the subject is permitted to work until fatigue. In the main, however, the temperature curves have the same shape. Fig. 10, subject P. H., shows the results of experiments with 3 different loads. The temperature rise in the work period is less steep at small loads, but the greatest difference is

found in the restitutional period, the temperature in the experiments with the small loads rapidly reaching the 0-line, while the 5-kg curve, which in this subject represents a considerable effort, after 15 minutes still is 1.2° above this line.

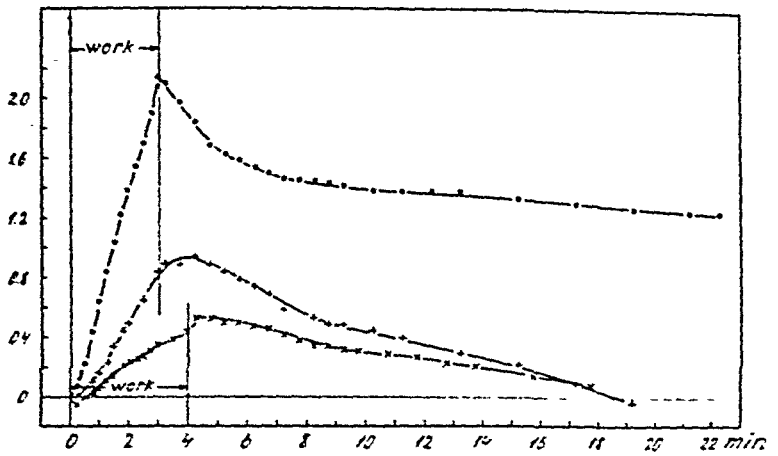

Fig. 10. P. H. Dynamic work with different loads. Upper curve 5 kg, middle curve 2.5 kg, and lower curve 1 kg.

If the experimental time varies, while the load is kept constant, it is found that the slope of the temperature curve during the work period is the same in all cases. Fig. 11, subject P. H., load 5 kg, duration of work 3, 1.5 and 0.5 minutes respectively. While

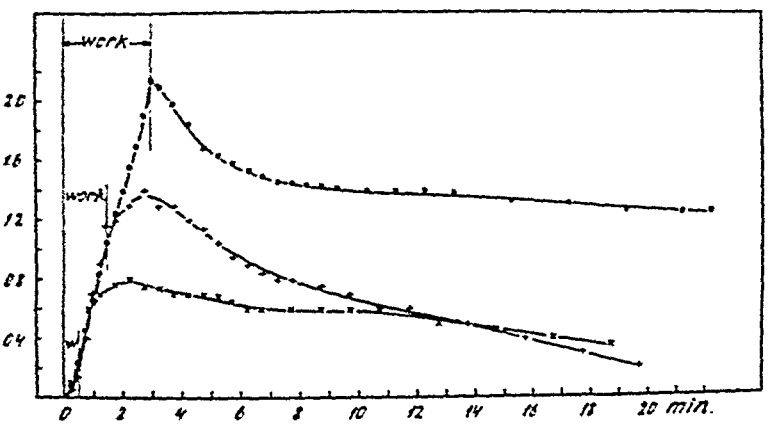

Fig. 11. P. H. Dynamic work. Load 5 kg. Duration of work 3, 1.5 and 0.5 minutes respectively.

the temperature after 3 minutes work immediately falls, the work of 1.5 minutes duration is followed by a slight rise in the after-period, and if the work lasts only 0.5 minute it is followed by a very sudden and strong temperature rise which gradually changes

into a slow fall. Since there can be no question of overloading or fatigue in the last mentioned experiments, it must be assumed that the temperature rise after the work is due to the circumstance that the regulation of the circulation has not reached an

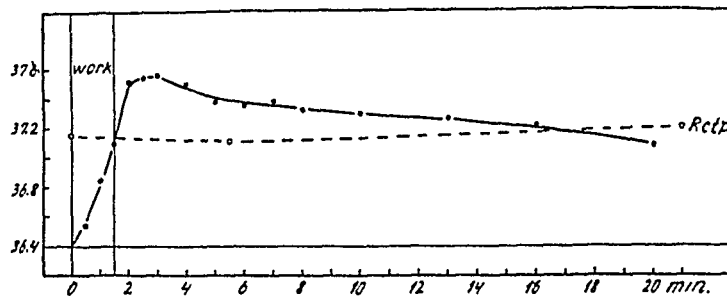

Fig. 12. F. B. Dynamic work. Load 10 kg. Temperatures directly recorded. The rectal temperature recorded in the figure.

adequate development in the short time that the experiment has lasted.

In experiment fig. 12, subject F. B., load 10 kg, working time 1.5 minute, we find conditions corresponding to the lower curve in fig. 11, with the sole difference that in this case, owing to the heavier load, all dimensions are larger. In this case the muscle temperatures are corrected, and the rectal temperature is recorded in the figure.

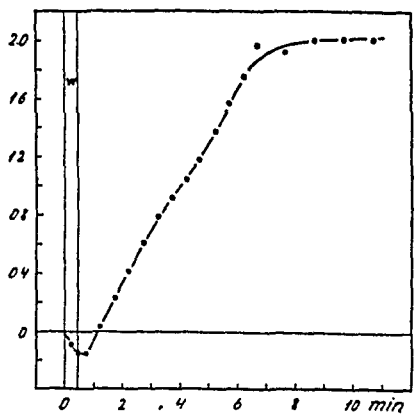

Fig. 13. P. H. Dynamic work. Load 10 kg. Zero-line = 34.4°.

This is shown rather strikingly in the following experiment, fig. 13. Subject P. H., load 10 kg. In this case the load exceeds the strength of the person, and the work time is thereby *forced* down to a minimum. After 0.5 minute of work we find a temperature fall of about 0,2°. From this point the temperature rises rapidly and reaches in 8 minutes 2° above the initial temperature. This level is maintained during the next 2 minutes, whereupon the experiment is stopped.

The temperature fall during the beginning of the work, which we find in several experiments and which in some is so pronounced that the experiment is rejected, must in all probability be due to

a dislocation of the thermo-needle in the muscle, the muscle having changed its shape during the contraction. When, as in fig. 13, the muscle is overloaded there is moreover apt to be a slight twisting of the arm which may cause a pull in the thermo-needle. It is highly improbable that the thermo-needle, once it has been dislocated, will return to its original position, but the dislocation will, if the needle remains in its new position, only cause a displacement of the initial value, but not affect the shape of the rest of the curve. In this connection it should be remembered, however, that a strong muscle contraction may cause a rather effective arresting of the circulation, and it is perhaps not impossible that this may in a few cases cause a slight fall in the muscle temperature.

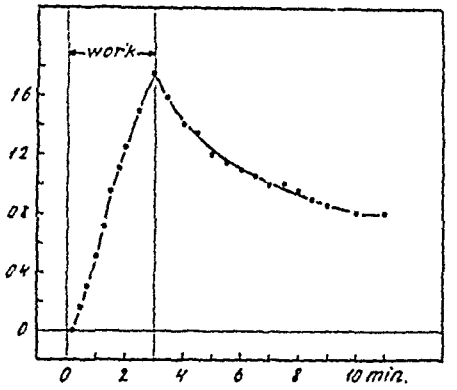

Fig. 14. F. B. Dynamic work. Load 5 kg. Zero-line = 35.8°.

In the above mentioned experiments with P. H. we find, during the dynamic work for 3 minutes with a load of 5 kg, an average temperature rise in the biceps of 2.05°. In stronger or better trained subjects the temperature rise is smaller. Thus in the case of F. B. we find in a 3 minutes experiment with the same work a rise of only 1.75°, *i. e.*, 15 per cent less than in the case of P. H. (fig. 14). In K. H. (fig. 15) the temperature rise after 5 minutes work is 2.2°, but after 3 minutes the temperature has risen only 1.4°, *i. e.*, 32 per cent less than in P. H.

In work of this magnitude the temperature falls immediately after the work has ceased. In K. H., however, it falls very slowly after 5 minutes work; only 0.5° in the first 5—6 minutes.

If one of the better trained subjects is given work to which he is not accustomed, the temperature rise becomes greater. In work with the dorsal flexors of the foot F. B. reaches in 2.5 minutes a temperature rise of 2.5°, which in the restitutional period increases to 2.75° (fig. 8).

Since the height to which the load is raised is about 10 cm with a timing of 34 contractions per minute, the total work in 3 minutes is $0.1 \times 5 \times 34 \times 3 = 51$ kgm. For this work we find the following temperature increases:

Subject	2 min.	3 min.	5 min.
P. H.	1.45	2.05	
F. B.	1.25	1.75	
K. H.	0.95	1.40	2.20°

Thus the slope of the curve after 2 minutes is about the same in the case of all 3 subjects. This, however, tells us nothing regarding the magnitude of the heat production that has occurred in consequence of the work, and this quantity cannot be calculated on the basis of the available data. The biceps is only one of the muscles of which the working synergy consists, and it is not

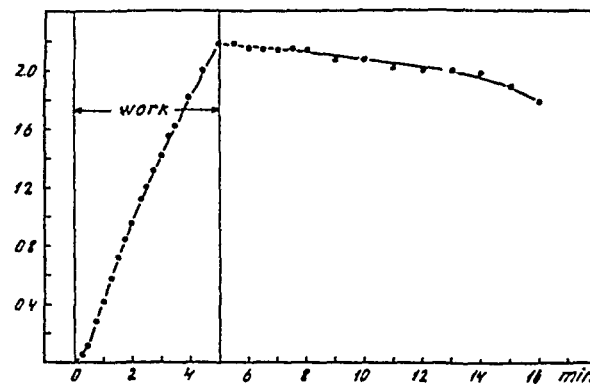

Fig. 15. K. H. Dynamic work. Load 5 kg. Zero-line = 33.8°.

known how the temperature in the other muscles changes during the work. It is probable that the temperature rise in, *e. g.*, brachioradialis will prove to be less, while in brachialis anterior it undoubtedly will be greater than in biceps. Moreover, in several cases heat will be carried away by the blood.

Static Work.

If we consider the muscle function from the point of view of the physiology of the muscle there is only one form for muscular activity, but, under changing conditions, this may manifest itself in different ways. According to the contraction theory which is developed in this laboratory, the molecular chains of the myosin micellae are extended beyond their equilibrium position by electrostatic forces, probably localized to the molecule itself. When the fibre is stimulated, a drop in the electrical potential occurs, which causes the contractile elements to approach their

equilibrium position. Then the electrical potentials will rapidly be reformed, and the fibre will resume its original length. If the fibre is not under load, the process will be reversible. But if the fibre is under load, and thereby prevented from attaining its equilibrium length, a mechanical tension will arise, and the fibre will be able to do mechanical work. If the tension developed is just in equilibrium with the load, no movement will occur, and thus no work will be done. If the tension is greater/smaller than the load, a positive/negative work will be carried out. Thus far there is no physiological difference between dynamic and static work. When the two "work forms" in practice behave differently, or *can* behave differently, it is because of the circulation in the muscle, *i. e.*, the working conditions. It is found that a strong static contraction can apparently arrest the circulation to the muscle almost as effectively as the application of a tourniquet. Hence the pronounced feeling of fatigue which soon makes itself felt in static work. These questions have been elucidated through determinations of metabolism (respiration experiments). The temperature measurements appear to give results which are easily brought into harmony with these findings.

It is unfortunate that a comparison between magnitude of work and intensity of work in the case of static and dynamic work cannot be made. Dynamic work can be expressed in any kind of work unit, but not so with static work. HARTREE and HILL (1921) have proposed to characterize the static activity of the muscle by the product: tension × time (tension-time), but that does not get us much farther, since the two quantities, work and tension-time, do not have the same dimension. As the responsible process in both instances is the same, it should be possible to obtain a common measure in the energy consumption, measured by the oxygen intake. But this way out is of no value here. One reason is that the magnitudes of the work concerned are so small that it would require a very long series of experiments to establish the rise in oxygen consumption with reasonable accuracy. Moreover — and this difficulty has so far proved insurmountable — it is impossible to keep the two forms for work separate. The dynamic work requires that the part of the body which is not moved during the performance of the work must be stabilized, and the stabilization takes place by means of static work. *A priori* it is a fact that the work of stabilization will be different when a weight is raised and lowered rhythmic-

ally and when the same weight is held in some fixed position; therefore it is impossible to use the oxygen consumption in the two cases as a measure of the energy transformation of a certain group of muscles. How much this stabilization work means energetically one may judge when considering that in work on a bicycle ergometer it is possible to achieve a mechanical efficiency of 20—25 per cent, while this efficiency is about 2 per cent when maximal work is done by the flexor muscles of the elbow. In the former instance the stabilizing work is quite small, in the latter very large. This extra work naturally entails a considerable heat production in all the active muscle groups, but this does not directly affect the temperature in the muscle group the work of which is measured in the given case; but if it is sufficiently large

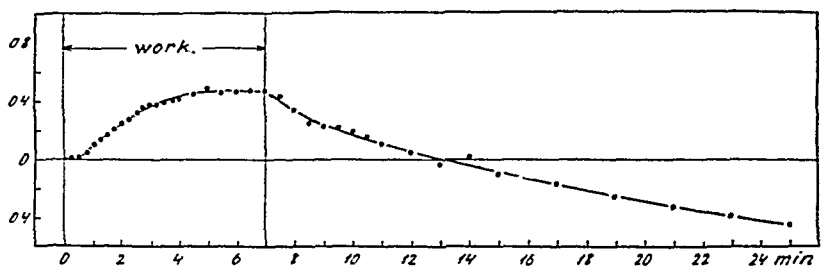

Fig. 16. P. H. Static work. Load 2.5 kg. Zero-line = 34.7°.

it must become manifest as a rise in the rectal temperature. When it has been impossible to demonstrate any such rise in our experiments it must be because the total evolution of energy due to the work is short-lived and relatively small.

Just as the dynamic muscle activity, when the tension is high and the contractions follow in rapid succession, may act like the static one, because the circulation is retarded by the strong tension and cannot get time to adjust itself in the short free intervals (fig. 13), or in cases of moderate but short-time work where the regulation of the circulation lags behind (fig. 12), so it will be found that very light static work may act as dynamic work.

Fig. 16, subject P. H., load 2.5 kg, work time 7 minutes, mean curve from 2 experiments. The muscle temperature rises only 0.45°; it falls immediately as the work ceases, after 6 minutes reaching the initial value, whereupon the fall in temperature continues evenly as at rest. During the last 3 minutes of the work the temperature seems to have reached some kind of "steady state".

Fig. 17, subject Chr. P., load 10 kg, mean curve from 3 experiments. A point on the curve thus represents 6 temperature measurements. The very strong subject has held the 10 kg for 4 minutes, with a rise in temperature of 1.12°; in the after-period the temperature rises only 0.1°; it reaches the 0-line in 22 min-

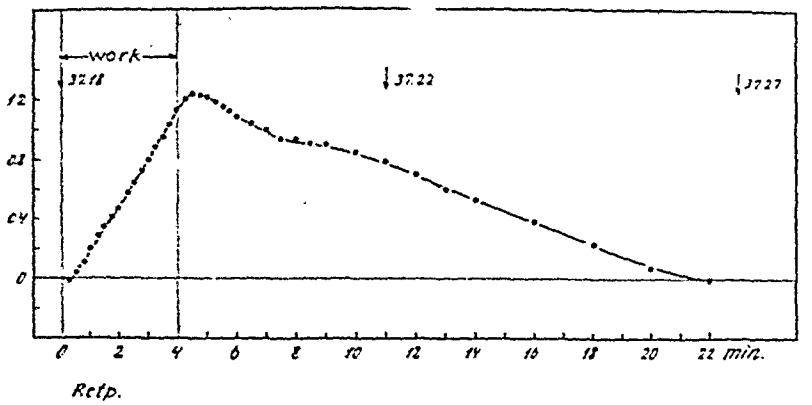

Fig. 17. Chr. P. Static work. Load 10 kg. Each point is the mean value of 6 temperature measurements. The rectal temperature is given at 3 different times Zero-line = 35.6°.

utes. This curve too is not clearly "static". The initial temperature is 35.6°, maximum 36.82°, corrected 37.32°. The rectal temperature at this time is 37.2°. It may be added that this is the only case in which we can demonstrate a slight rise in the rectal temperature during the whole experiment.

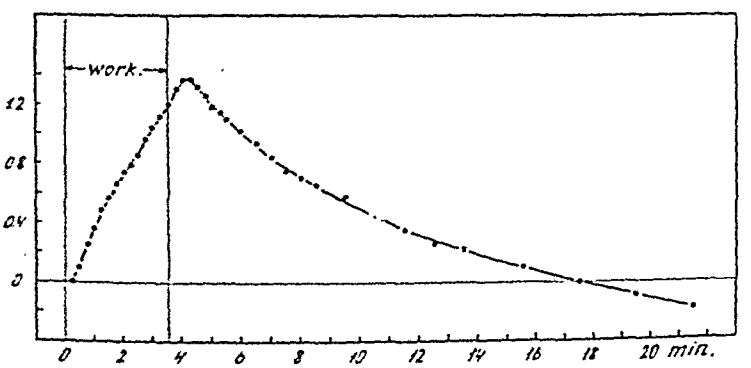

Fig. 18. F. B. Static work. Load 10 kg.

The curve in fig. 18 shows a similar course. Mean values from 2 experiments, subject F. B., load 10 kg. During the work period the temperature rises 0.95°, and reaches in the after-period, in the course of 1 minute, 1.25° above the initial value, whereupon it slowly falls.

In these experiments we find once more that the strongest person has the lowest temperature rise when the work is the same.

Subject	2 minutes	3 min.	4 min.
F. B.	0.56	0.95	
Chr. P.	0.46	0.79	1.12°

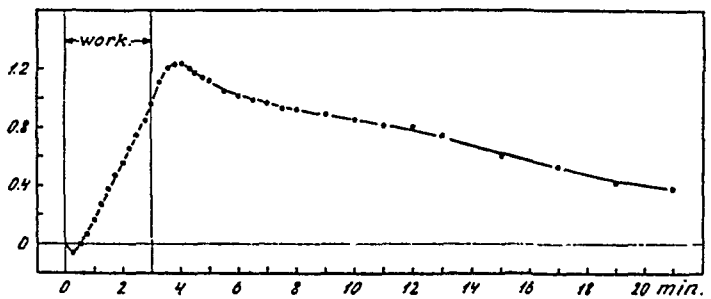

Fig. 19. P. H. Static work. Load 5 kg. Zero-line = 34.8°.

In fig. 19, subject P. H., load 5 kg, the temperature rises 1.2° during the work and reaches in the first minute of the restitutional period 1.4°, whereupon it falls rather rapidly. By comparing this temperature rise with the rise during dynamic work with the same load (fig. 9) it is found that the rise in static work is only about half of what it is in dynamic.

P. H., load 5 kg.	2 min.	3 min.
Dynamic work	1.45	2.05
Static work	0.74	1.05°

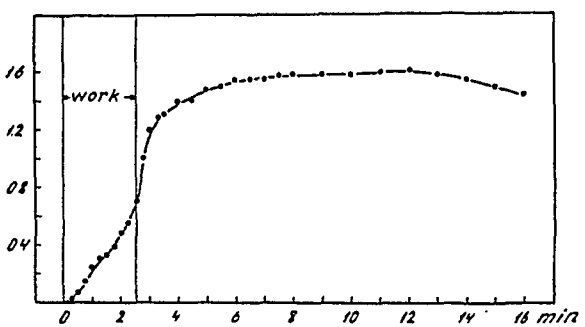

Fig. 20. K. H. Static work. Load 10 kg. Zero-line = 34.0°.

Fig. 20, subject K. H., load 10 kg, is an example of a typically "static" curve. The subject has been able to hold the weight for 2.5 minutes, and during this time the temperature has risen 0.7°. After the work has ceased, however, the temperature rises

rapidly, then again very slowly, reaching 1.6°, 10 minutes after the work has stopped. Thereupon the temperature shows a slow fall, and 4 minutes later it is still 1.45° above the initial temperature.

A comparison between fig. 20, the purely static work, and fig. 21, dynamic work during *arrested circulation*, shows, what was to be expected, that it is the failing circulation through the statically contracted muscles that is responsible for the shape of the "static" curve. Fig. 21 illustrates an experiment with K. H., dy-

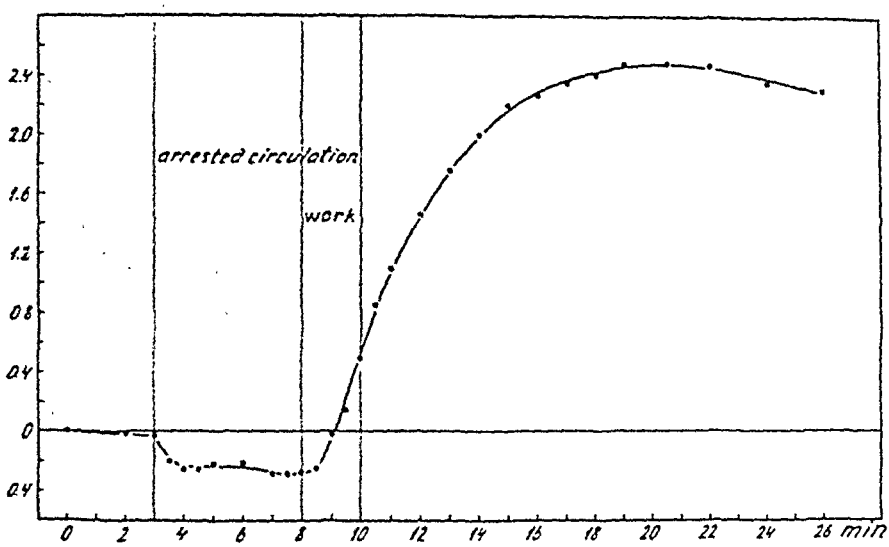

Fig. 21. K. H. Dynamic work. Load 5 kg. Circulation arrested before and during the work.

namic work, load 5 kg. During rest, with the circulation arrested, before the work, the temperature in the muscle falls 0.3°; then follows a rise during work of 0.8°. When the circulation is restored simultaneously with the cessation of work, the temperature rises strongly and reaches in 10 minutes a maximum of 2.5° above the initial temperature. It then falls slowly, only 0.2° in the next 6 minutes.

In the following experiments the arresting of the circulation is continued for a few minutes after the work has ceased. Figs. 22 and 23 again present opportunities for comparison between static and dynamic work.

Fig. 22, subject P. H., static work, load 5 kg. The circulation arrested. Each point on the curve is the mean of 4 readings. Immediately after the application of the tourniquet the muscle

temperature falls and reaches — 0.6°; during work in 3 minutes the temperature rises about 0.1°. After the work has ceased there follows a slight rise, and then a fall to — 0.7°. At this time the circulation is restored, and the temperature rises in 3.5 minutes

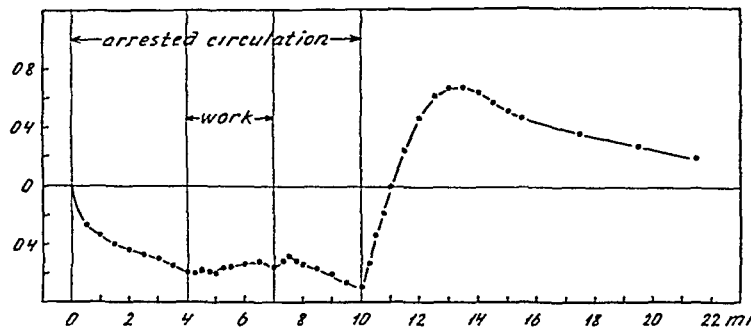

Fig. 22. P. H. Static work. Load 5 kg. Circulation arrested before, during and after the work. Zero-line = 35.1°.

to a maximum of + 0.7°, then falling evenly towards the initial temperature. Compare fig. 4, an experiment at rest with the same subject, where the circulation has been arrested for 10 minutes.

Fig. 23 records an experiment with K. H., dynamic work, load 5 kg. In all essentials this curve has the same shape as fig. 22. It

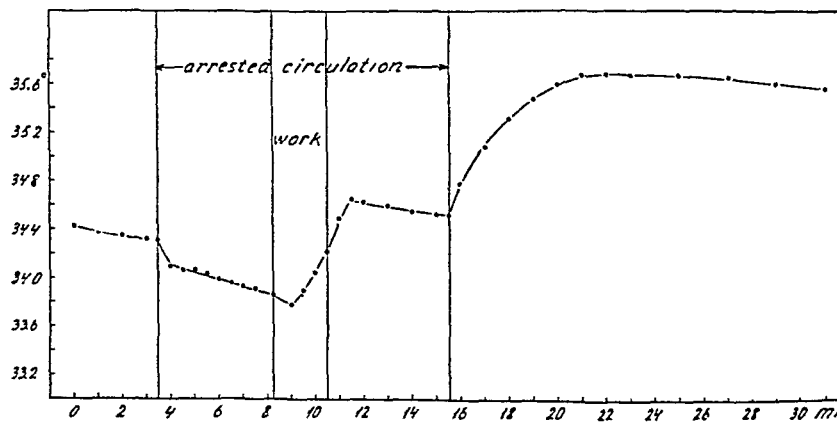

Fig. 23. K. H. Dynamic work. Load 5 kg. Circulation arrested before, during and after the work.

is observed that at the beginning of the work there is a small temperature fall, which might indicate a dislocation of the needle electrode; moreover, that the temperature rise during the work in this case is considerable, and, finally, that the temperature

rise in the restitutional period is greater and is maintained much longer.

In another experiment with K. H. (static work, load 10 kg; circulation arrested before the work was started) during work the temperature rises 0.8°. In the first minute thereafter it shows a further rise to 1.0°, then falling a little, until the circulation, 6 minutes after the work is discontinued, is again restored. At this time there begins a strong temperature rise which reaches a maximum, 3.1° above the initial temperature, 11 minutes after the work has ceased. 5 minutes later the temperature is still 2.9° above the initial temperature.

The experiments with work while the circulation is arrested all show that the temperature in the muscle rises during the work, even though the circulation has been arrested for several minutes prior to the start of the work. This observation, which has previously been made by LIPPROSS (1942), can only mean that during the contraction there occurs non-oxidative, heat producing processes in the muscle to a not insignificant extent. It seems reasonable to attribute the passing temperature rise, which occurs immediately after the work has ceased, to this heat production. This continuation beyond the work period should be characteristic of the anaerobic work. One might perhaps ask: Is it not possible that the small temperature rise during arrested circulation *after* the anaerobic work can be due to a dislocation of the needle electrode in the muscle, in analogy with the previously demonstrated temperature fall at the beginning of the work which has been observed in a couple of the last mentioned experiments? Such an assumption is rather improbable, however. In the cases concerned it is not a matter of a very sudden, brief temperature fall, but a temperature fluctuation lasting 1—3 minutes. Moreover, it is highly improbable that a termo-needle which has been dislocated should find its way back to its original position; it is easy to understand that a needle may perhaps slide out, but it is difficult to see how it should slide in again.

The Rectal Temperature.

In the experiments the rectal temperature has varied from 36.8° to 37.4°, in most cases being between 37.0° and 37.2°. As a general rule it is found that when a subject occupies a reasonably

comfortable sitting position, the temperature will fall quite evenly and slowly. This we have observed in several experiments. In other instances the rectal temperature has remained constant during the experiment, or there has been small fluctuations, purely accidental in character. Only in one subject (see fig. 17) have we been able to demonstrate an even, but slow rise in the rectal temperature, which presumably must be attributed to the work. Of course, it is quite probable that, in the experiments where the rectal temperature has remained constant, one may see an effect of the work just compensating the fall which otherwise might have occurred in consequence of the prolonged sitting still of the subject.

When the demonstration of the influence of the work on the rectal temperature is so uncertain it is undoubtedly because the work intensity is so small, and also because the period of work is so short. 17 kg/min. is a very small work intensity, even though supplemented by the unavoidable stabilization work, and 2—5 minutes is a very short work time. It was impossible, however, to increase these quantities, since the synergy used could not produce any more.

Discussion.

~~Numerous experiments~~ have shown that the body temperature, the rectal temperature, rises during muscular work. Such experiments have in particular been carried out by means of the bicycle ergometer, where both the intensity and the duration of work can be made many times greater than it was possible in our experiments. The experiments with which we are dealing show that the muscle temperature also increases during work, and that this rise indicates a production of heat in the muscle itself. The temperature in the extremities is, as mentioned above, considerably lower than the rectal temperature, though there is a continuous flow of blood through them, with a blood temperature which, before reaching the extremities, is at least equal to the rectal temperature. This fact can hardly be explained otherwise than by assuming that the blood, owing to the relatively large surface of the extremities, is exposed to cooling in excess of that circulating in the body. If we imagine that, at unchanged heat production, more blood flowed to the extremities, then this would at first cause some rise in the temperature of the extrem-

ities, but the rise would be slow. The rate of flow of the blood, which in aorta may be estimated to be 11—12 cm/sec., decreases gradually towards the periphery and is very low in the capillaries, and these, even in a strongly vascularized skeletal muscle, constitute but a fraction of the mass of the muscle. The heating of the muscle in this manner would at the same time cause an increased fall in the rectal temperature. Unfortunately, it is hardly possible to determine the temperature movements quantitatively, because we do not know the quotient of the total blood that passes to an extremity, nor the part of this that passes to the muscle investigated, whether at rest or during muscular work. However, *a priori* it is not probable that an extremity muscle should be heated several degrees, solely because of an increased flow of blood, unless an increased heat production occurs at one place or another, nor unless the rectal temperature shows a considerable fall. If in some organ there occurred a considerably increased production of heat, then this would manifest itself as an increase of the body temperature, and such an increase would, *ceteris paribus*, also manifest itself in the form of a rise in the muscle temperature. But this rise would be accompanied by a still greater increase of the rectal temperature, and no such rise has been found in our experiments. Hence it is improbable that chemical processes should occur in the working muscle, causing the formation of substances which, not in the muscle but in another organ (*e. g.*, the liver), might give rise to metabolic processes with positive heat effects. Such an assumption is not only improbable, but impossible in the cases where the temperature lies above the rectal temperature. Moreover, it is not in the case of heavy work that the muscle temperature exceeds the rectal temperature — it is mainly in the experiments where the initial temperature is high. In fig. 17, subject Chr. P., which represents the heaviest work done in our experiments (the other test persons being unable to perform it) the maximum of the muscle temperature exceeds the rectal temperature by only 0.13°. In subject K. H., who was thin but rather muscular, and who had low muscle temperatures at rest, the temperature in the muscle never reached the rectal temperature, though the subject showed large temperature rises during the experiments. In F. B., whose muscle temperature at rest was high, with an average of about 36°, the muscle temperature in several instances rose considerable above the rectal temperature. For example:

Fig. 12. Initial temperature (corr.) 36.4°. Rise during experiment 1.16° = 37.56°. Rectal temperature 37.13°.

Fig. 7 a. Initial temperature (corr.) 37.2°. Rise during experiment 1.05° = 38.25°. Rectal temperature 37.25°.

When the temperature in the muscle, as in the experiment 7 a just mentioned, at the start of the experiment is very close to the rectal temperature, it is out of the question that the temperature rise in the muscle can be due to increased circulation, and since we cannot assume that the initial temperature can influence matters of fundamental importance, it is necessary to assume that the circulation in all cases plays a secondary rôle in this connection, even though in cases with very low initial temperature one may imagine a temperature rise due to increased circulation. It is the temperature of the blood in the supplying artery which determines whether the muscle is to give off or to receive heat during the circulation. We do not know this blood temperature, but it must be assumed from the present investigation that it is lower than the rectal temperature. Hence we are justified in assuming that the temperature rise in the muscle during work is due to heat-producing processes in the muscle itself.

On the basis of investigations on the temperature regulation during muscular work, showing that the heat regulation does not adjust itself as one would expect if the object was to reduce the body temperature, NIELSEN (1938) has shown that the increase of the body temperature which occurs during the work, and which appears to be proportional to the magnitude of the work, is to be considered as a measure of expediency, the higher temperatures being taken to accelerate the metabolic processes in the muscle. For work of the order of magnitude which occurs in our experiments the rectal temperature is not to be taken into account, and even in experiments involving heavy work which has strong influence on the rectal temperature, the rise in temperature is so slow that its significance to the work in many instances may be illusory. But when the temperature rise occurs in the working muscle itself, and begins simultaneously with the work then we have conditions where the increased temperature may act as assumed by NIELSEN. Thus our experiments support his hypothesis.

Inasmuch as the temperature curves on the whole follow a course similar to that of oxygen absorption curves, it would seem reasonable to conclude that the temperature rise in the muscle

is mainly due to oxidation processes. The function of the circulation is to supply the muscle with oxygen, and not to heat the muscle. The temperature rise during work, with the circulation arrested, shows, however, that anaerobic heat-producing processes may occur in the muscle, the muscle being unable to store oxygen. Also in the cases where the arresting of the circulation extends beyond the cessation of work do we find a small, rapidly passing, rise in the temperature after the work. This leads to the idea that also the non-oxidative, heat-producing processes in the muscle are associated with the restitutional phase of the muscle fibre and not with the contractional phase.

Summary.

1. The temperature in resting muscles, measured by means of needleshaped thermocouples, shows considerable variation. In the muscles of the extremities it is most generally 34—35.5°. In the thenar the temperature is found to be 4° lower than the rectal temperature.

2. When the subject is sitting still, with the skin exposed, the muscle temperature shows a gradual fall.

3. During dynamic work (51 kgm in 3 minutes) with the flexor muscles of the elbow, the temperature in the biceps rises 1.4—2.05°, the least in the strongest subjects. In the after-period the temperature falls gradually, the faster the less the work has been. Even relatively light work may, however, occasionally lead to protracted displacements of the temperature level in the muscle.

4. For the same subject the temperature rise during the work, with the same work rhythm, is directly dependent upon the magnitude of the work.

5. In experiments of very short duration the muscle temperature rises after the work has ceased, presumably because of inadequate circulation during the work.

6. In dynamic work with heavy load the muscle temperature is found to behave as in static work; conversely, in static work with light load the temperature will behave as in dynamic work.

7. During static work the muscle temperature rises moderately or only very little during the work period, but the rise continues for a shorter or longer period after the work has ceased.

8. This phenomenon is still more pronounced when the circula-

tion to the working muscle group is arrested. Then the high temperature rise will not appear until the circulation is restored.

9. The experiments show that the temperature rise in the muscle in the main is due to processes with positive heat effect in the muscle itself, not to increased circulation.

10. The heat production is mainly due to oxidation processes, but is also observed during and after work with the circulation arrested. The anaerobic heat production is very small, however.

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The Phytin Contents of Norwegian Flour and Bread Types I.

The Phytin Contents in the Usual Flour Types and the Rate of Decomposition in Flour Suspensions.

By

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Phytin, or more correctly phytic acid, is a hexaphosphoric acid ester of inositol and forms in pure state a yellowish, clear, viscous liquid. Usually, however, the word "phytin" means salts of the phytic acid, of which a great number is known. In nature we find neutral Ca-Mg double salts, insoluble in water, while the trade preparations are acid salts, more or less soluble. The Fe, Cu, Pb and Ag salts are insoluble and can be used for the isolation of phytin. The natural Ca-Mg salts are soluble in diluted acids without decomposition and can be extracted for instance with n/2 HCl.

The enzyme phytase splits phytin into its components, inositol and inorganic phosphate. ADLER (1915) states that phytase has its pH-optimum about 5.4 and its greatest activity from 43 to 58° C, above which temperature it is very rapidly spoiled. PEDERSEN more recently has found the optimal pH about 5.2. In malt, according to ADLER, at least two types of phosphatases are present, one of which brings insoluble organic P into solution, while the other performs further splitting to inorganic phosphates.

In plant seeds, especially the cereals, the greater part of the total phosphorus is present as phytin. No phytin, however, is found in vegetables and greens. (ARBENZ 1922). The physiological

importance of phytin had been very differently judged. Some scientists, especially in the years up to 1920, have regarded it as a phosphorus source of great value, and phytin has been produced industrially in pure state for medical purposes. Other investigators, however, maintain that the resorbtion of phytin as such is very doubtful, and that no resorbtion can take place unless it has been split by phytase into its components.

This splitting can be performed during the preparation of the food, or at last in the intestine by phytase accompanying the food, but the presence of phytase among the digestive enzymes is doubtful, and if pure phytin is given, most of it will be found uninjured in faeces. Thus, in food lacking of phytase, we cannot expect any splitting of phytin, and then, if we calculate with the total phosphorus present, the apparent Ca : P balance will give a false picture. Under given conditions, this balance can be so disturbed that rachitis will result. This view is greatly supported by the work of PEDERSEN (l. c.) through his experiments with pigs. In his publication, a very complete survey of the literature on phytin is also given.

Among the cereals, oats and maize are reputed to be rachitogenic, and various scientists in vain have been searching for a "toxic" factor in these products. It appears that the rachitogenic properties of oats and maize are due not to the presence of a toxic factor, but to the lack of another factor, namely phytase. One cannot discount the possibility that, under certain circumstances, these cereals may have a similar effect in human nutrition, and thus an examination of the food articles on cereal base is justified. To some extent such examinations are referred by PEDERSEN but a more complete survey is desirable, partly because the baking methods may differ quite much from one country to another, giving different chances for the enzymatic breakdown of the phytin, and especially because in Norway during wartime oat meal is mixed into the ordinary bread flour, sometimes in considerable quantities.

In the present paper, then, the phytin contents of a number of the usual types of flour is examined together with their ash and protein contents, and then follows a study of the rate of decomposition of the phytin due to their own phytase contents in water suspensions. The corresponding decomposition in doughs under various conditions and the remaining phytin in the usual bread types will follow in another paper.

Methods.

Phytin can be extracted from cereals etc. with n/2 HCl, which will also inactivate the phytase present. A titrimetric precipitation method for the determination of phytin is given by HEUBNER and STADTLER (1914). The method, further elaborated by ARBENZ, is based upon the precipitation of phytin with a $FeCl_3$-solution in 0.6 % HCl, since inorganic phosphates, simultaneously present, are not precipitated as ferric salts by this acidity. NH_4CNS serves as indicator, and a faint rosa ("flesh-coloured") marks the endpoint of the titration. The multiplication of the number of milligrams Fe with 1.19 is said to give the amount of phytin-P. This procedure, however, now is replaced by better methods, since the repeated extractions recommended are time-wasting, and the endpoint of the titration is uncertain, especially in the presence of much inorganic phosphate.

The ferric phytate precipitated in a cold fluid is colloidal, but it can be brought to flocculate through heating in a boiling water bath for 15 minutes, thus permitting a quantitative separation from the liquid. For determination, two ways can be followed:

YOUNG operates with a definite quantity of $FeCl_3$ and determines the excess of Fe colorimetrically in the resulting liquid, from which the iron in the precipitation and thus the phytin P can be calculated.

MCCANCE and WIDDOWSON (1935) decompose the ferric phytate with NaOH, filter off the ferric hydroxide, ash the resulting Na-phytate with $H_2SO_4 + HNO_3$ and determine phytin P herein.

The latter method, described in detail by PEDERSEN, has also been adopted by the author, who has simplified it to some extent. The procedure is as follows:

The bread or flour extract, normally 10 gms. of flour in 100 ml n/2 HCl, is centrifuged. 50 ml of the liquid is neutralized with 5-n NaOH and filled up to 100. 50 ml of this plus 10 ml of $FeCl_3$ solved in n/1 HCl are heated for 15 min. in a boiling water bath. After cooling the liquid is centrifuged, whereby the ferric phytate forms a coherent mass on the bottom of the glasses, permitting a complete decantation of the liquid. The precipitation now is decomposed with 20 ml n/2 NaOH, transferred to the large test tubes in which the first heating took place, and heated again in the water bath for coagulation of the ferric hydroxide. The liquid then is brought back to the centrifuge glasses, washing after with water, and centrifuged. The Na-phytate solution is decantated into Kjeldahl flasks for mineralization with nitric plus sulphuric acid, and P determined herein. The ferric hydroxide can be solved in H_2SO_4 for determination of Fe, if wished, either colorimetrically, or the ferric sulphate is reduced to the ferro compound with pure zinc powder and titrated with $KMnO_4$. In a great number of such double determinations, the author has found a ration phytin P : Fe of 0.75 instead of the factor 1.19 as reported by HEUBNER- STADTLER and ARBENZ. The explanation must be that the ferric phytate is not completely precipitated by the titration method of these authors, so

that the amount of Fe bound at the endpoint is too small. In most recent investigations, however, the phytin phosphorus has been determined as such, so that the results reported can be regarded as valid.

Contents of Total P and Phytin P in Flours in Relation to Their Ash and Protein Contents.

Data for several flours are given in the subsequent table 1. All numbers are calculated for dry matter and expressed as grams in 100 grams. The phytin contents of the flours is seen to increase with the ash. Since this again is related to the degree of extraction of the flour, it means that the phytin is present chiefly in the outer layers of the grain, bran and germ. This is in accordance with earlier findings, for instance by PRINGLE and MORAN (1942). No relation can be found between phytin and protein contents of flour, and such a relation is hardly to be expected. In flours with a high mineral content, phytin P makes a higher percentage of total P than in white flours with little ash. Generally the total phosphorus of flours, expressed as P_2O_5, forms about 50 % of the ash. According to PRINGLE and MORAN, the amounts of Ca

Table 1.

Contents of ash, protein, total and phytin P in various flours.

Flour	Ash	Protein	Total P	Phytin P	Non-phytin P	Phytin P % of total P
Wheat flours:						
Norwegian, 73 %	0.87	11.1	0.195	0.091	0.104	46.5
Flour of Central-European wheat .	0.88	9.5	0.188	0.108	0.080	57.5
Do. ca. 85 % . . .	1.15	10.8	0.255	0.177	0.078	69.5
Wholemeal 94 %, American wheat .	1.96	18.6	0.467	0.332	0.135	71.0
Wholemeal, Norwegian wheat . . .	1.90	11.2	0.453	0.320	0.133	70.7
Rye flours:						
White flour 67 % with 15 % wheat .	0.84	7.3	0.184	0.099	0.085	54.0
Do.	0.87	7.1	0.145	0.077	0.068	53.0
Flour ca 82 % . .	1.18	8.7	0.264	0.166	0.098	63.0
85 % flour from sprouted rye . .	1.60	10.7	0.332	0.149	0.183	45.0
Whole meal 94 % .	1.68	9.0	0.370	0.269	0.101	72.7
Barley flours:						
58 % flour	1.50	9.2	0.376	0.225	0.151	60.0
80 % »	1.86	10.8	0.415	0.296	0.119	71.5
80 % »	1.92	10.0	0.447	0.306	0.141	68,5
Oat meal, 60 % . .	2.24	11.3	0.482	0.255	0.227	53.0

and Mg in whole wheat meal are sufficient to bind the total phosphorus as a mixed Ca-Mg-phytate, and this would suggest a high correlation between ash content and phytin P in flours. This is in fact confirmed by the author's results, and further evidence is given by PEDERSEN. In his publication ash values are not given, but under the condition that P_2O_5 forms 50 % of the ash, the numbers can be calculated. In the graph figure 1

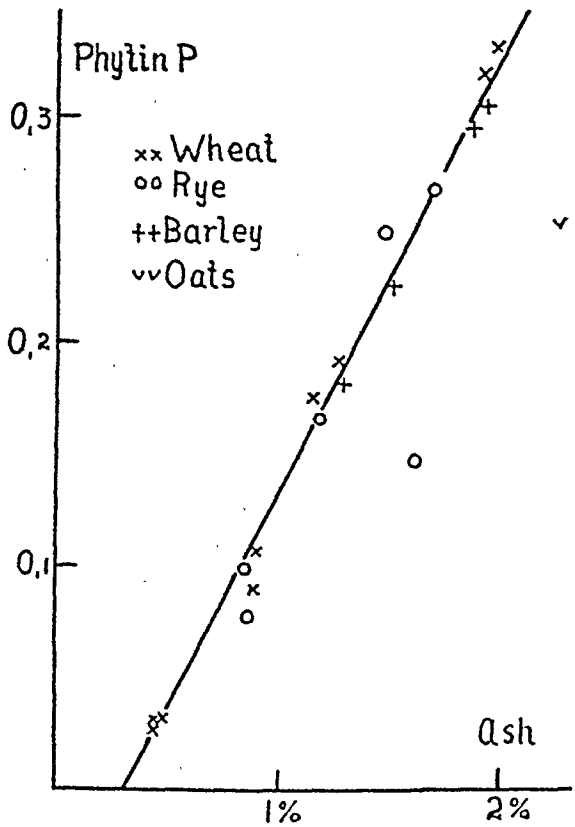

Fig. 1. Relation between ash and phytin contents of various flours.

the author's results are grouped with the values calculated from PEDERSEN's publication. It will be seen that the observations with very few exceptions are crowded about a straight line, which indicates that the phytin content will be reduced to nought in flours with the lowest mineral content attainable, about 0.30 g/100 g. In such flours, the total P approximately will amount to 0.065 g/100 g.

This amount is of interest when compared with the column "Non-phytin P" in table 1. Even if the quantities of Ca and

Mg present are sufficient to bind all P as phytin, there will always be a rest, which is not related to the ash content, and largely of the same size of order, about 0.065 g/100 g. This is confirmed by some of the results of PEDERSEN, cited by MÖLLGAARD, and referred here in table 2.

Table 2.

Total P and phytin P in Danish flours.

Flour	Total P	Phytin P	Non-phytin P
Wheat flour 1	0.095	0.028	0.067
» » 2	0.097	0.031	0.066
» » 3	0.100	0.033	0.067
Whole wheat meal	0.275	0.192	0.083
Wheat, whole grain	0.319	0.273	0.046

This non-phytin P consists of the phosphorus bound in proteins and lipoids, and further a small, but definite amount of phosphorus is always bound as amylo-phosphoric acid esters in the amylopectin of the starch.

In wholemeal, the amount of non-phytin P often is a little higher, but very few cases show a deviation which is really significant. One of them is an oatmeal, another is an 85 % extraction of a severely sprouted rye, so damaged that it cannot give a proper bread. It is possible, in this case, that a decomposition of originally present phytin has taken place during the beginning germination.

Rate of Decomposition of Phytin by Phytase of the Flours.

For the examination of phytase activity in flours, the following procedure was adopted:

A suspension was made of 100 gms. of flour and 300 ml water, saturated with toluene. Care was taken that the suspension at once obtained 25° C, at which temperature it was kept in an incubator. Various pH levels were adjusted by addition of lactic acid. At intervals samples of the viscous suspension were weighed for examinations. Remaining phytin was extracted by making the sample n/2 with HCl, further procedure as above. Besides, the quantity of phosphorus in solution was determined by diluting a sample of the suspension with cold water and centrifuging at once. 50 ml of the supernatant liquid was pipetted off for ashing with $H_2SO_4 + HNO_3$, and P determined herein.

In this way, a whole wheat meal, a 67 % white rye flour and a whole rye meal, a 80 % barley and a 60 % oat meal were studied at 3 or more pH levels, namely the natural pH of the suspension, about 5.2 and sourer (4.3—4.0). To save space, only one of the numerous analytical tables is given as an illustration (table 3).

Table 3.

Whole wheat meal, total P 0.453 g/100 g, phytin P 0.320 g/100 g, at pH 5.2.

Time min.	P in solution	Remaining phytin P	Decomposed phytin	P in solution ÷ decomp. phytin
30	0.235	0.230	0.090	0.145
60	0.296	0.153	0.167	0.129
120	0.348	0.0	0.320	0.028
180	0.371	—	»	0.051
240	0.390	—	»	0.070
1440	0.407	—	»	0.087

Remaining phytin P may be undissolved and dissolved, not yet decomposed. The latter part is included in the column P in solution, which for the rest consists of inorganic phosphates from the decomposed phytin, and organic phosphorus compounds others than phytin. The nature of this latter component is visible in the last column of the table. Here, we first find considerable quantities of phosphorus, doubtlessly consisting chiefly of undecomposed, dissolved phytin, which decrease with the progressive activity of the phytase. Then this column shows but small amounts of phosphorus, increasing slowly with time. In a sourer medium, the solubility is a little higher, but a little rest of the total P is always left undissolved. As expected, the solubility of undecomposed phytin is greater the sourer the suspension. The total picture of phytin decomposition and solution of phosphorus is given in figures 2 a-c.

As for the enzymatic decomposition of phytin, it has been found to have its greatest rate at pH 5.2, in accordance with PEDERSEN. For illustration, the times for 50 and 100 % decomposition for the flours are given in table 4.

The table illustrates quite clearly the pH effect on the enzymatic process, which is in very good accordance with the results of PEDERSEN. Besides, it is remarkable how much faster phytin decomposition takes place in rye flours, both in white and whole meal products, than in wheat and barley. This gives evidence

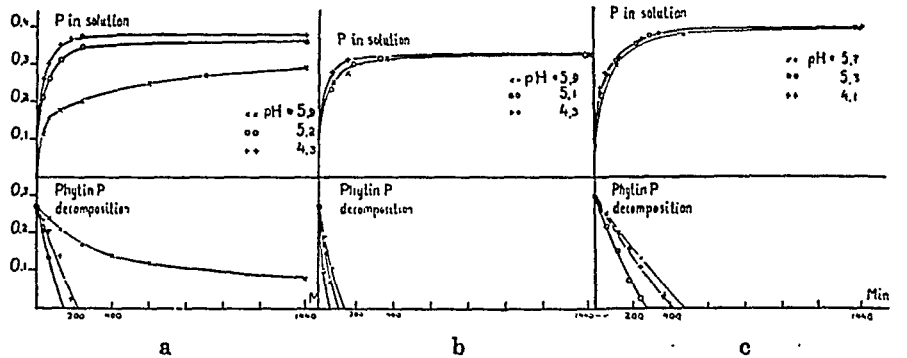

Fig. 2. Phytin decomposition and solubility of phosphorus in suspensions at various pH levels. a) whole wheat meal b) whole rye meal c) 80 % barley flour.

Table 4.

Rate of enzymatic decomposition of phytin at various pH levels.

Flour	pH	Phytin decomposition	
		50 %	100 %
Whole wheat meal . . .	5.9	450 min.	Decomposition not finished after 1440 min.
» » » . . .	5.2	55 »	120 min.
» » » . . .	4.7	105 »	210 »
» » » . . .	4.3	120 »	240 »
Rye Flour 67 % . . .	5.6	Under 30 »	
» » » » . . .	5.3	» » »	
» » » » . . .	4.0	25 »	55 min.
Whole rye flour	5.8	35 »	135 »
» » »	5.1	20 »	60 »
» » »	4.3	45 »	150 »
» » »	5.8	115 »	— Suspension kept at 10°.
» » »	5.15	70 »	150 min. »
Barley 80 % flour . . .	5.7	200 »	480 »
» » » » . . .	5.3	120 »	260 »
» » » » . . .	4.7	130 »	310 »
» » » » . . .	4.1	190 »	400 »

that the phytase activity in rye is much more powerful, and when PEDERSEN finds a more complete decomposition of phytin in rye bread that in wheat, even whole meal bread, it is not only a consequence of the different baking methods, but even more of the nature of the flours. When in the white rye flour the phytin has disappeared, even faster than in the whole meal, it cannot be taken as a sign of greater phytase activity, but gives only an expression that a fair amount of phytase has to split a much smaller quantity of phytin (0.077 g/100 g against 0.269 g/100 g.)

In two cases, suspensions of the whole rye meal were kept at 10° C, and here we find, as expected, that the decomposition is very retarded.

As for the oat meal no phytase activity is found, in full accordance with PEDERSEN, and the pH level is without importance. Therefore, the oat meal is not included in table 4. What is of interest in this case, however, is the rate with which phosphorus goes into solution, when the acidity is varied, and the results of these experiments are given in table 5.

Table 5.

Solubility of phosphorus from oat meal at various pH levels.

Time	pH 6.1	pH 5.7	pH 5.1	pH 4.3
30 min....	0.057 g/100 g	0.104 g/100 g	0.164 g/100 g	0.203 g/100 g
60 » ...	0.075 »	0.138 »	0.186 »	0.242 »
120 » ...	0.094 »	0.164 »	0.230 »	0.263 »
180 » ...	0.110 »	0.183 »	0.252 »	0.274 »
240 » ...	0.119 »	0.197 »	0.269 »	0.290 »
1440 » ...	0.139 »	0.235 »	0.307 »	0.318 »

Since no phytin is decomposed, the amount of soluble phosphorus evidently is determined by the solubility of the phytin as such. The meal had a total P content of 0.482 g/100 g, of which phytin P was 0.255 g/100 g. Supposed that all phytin had got into solution at the lowest pH, it will be seen that but very little of other phosphorus compounds were solved. These compounds make a much larger part of the total P in oat meal than in other flours, and it is striking how little of them which will be solved even in acid media — oats here shows a marked difference from other cereals.

Since oats during war time forms a part of the usual bread flour, mixtures of oat meal with various quantities of whole rye meal were examined, all of them at pH 5.1. The components and mixtures were as follows:

	Rye whole meal	75 % rye 25 » oats	50 % rye 50 » oats	25 % rye 75 » oats	Oatmeal
Total P.	0.370 g/100 g	0.400 g/100 g	0.415 g/100 g	0.447 g/100 g	0.482 g/100 g
Phytin P	0.269 »	0.268 »	0.272 »	0.262 »	0.255 »

In the mixtures, the following rates for phytin decomposition were found:

	75 % rye 25 » oats	50 % rye 50 » oats	25 % rye 75 » oats
50 % decomposition. . . .	30 min.	50 min.	110 min.
100 » »	80—90 »	130 »	250 »

Consequently, the phytase of rye is powerful enough to decompose the phytin of the oats within a reasonable time, even if only 25 % rye meal is added.

This is of practical importance if a decomposition is wanted, for instance in the feeding of pigs, and even in human nutrition.

The rate of solubility of phosphorus in suspensions of the components and the mixtures is given in table 6, pH for all suspensions 5.1.

Table 6.

Rate of solubility of phosphorus in rye and oat meal mixtures.

Time min.	Rye whole meal	75 % rye 25 » oats	50 % rye 50 » oats	25 % rye 75 » oats	Oat meal
30	0.171	0.218	0.208	0.202	0.165
60	0.231	0.258	0.248	0.252	0.187
120	0.303	0.290	0.282	0.282	0.230
180	0.316	0.300	0.300	0.292	0.252
240	0.323	0.308	0.310	0.300	0.268
1440	0.330	0.335	0.348	0.334	0.175
Undissolved P . . .	0.040	0.045	0.067	0.113	0.175
Do. calculated for the mixtures . .	—	0.075	0.108	0.144	—

The solubility decreases with increasing amounts of oats in the mixtures. The undissolved rest, however, is always smaller than expected, if calculated from the percentages of rye and oats. This seems to indicate that a part of the insoluble phosphorus of the oat meal is brought into solution by the rye, probably by some enzymatic process.

Summary.

Phytin has been determined in a number of commercial flours with the following results: With few exceptions, there is a very near relation between ash and phytin contents of the flours. The results indicate that the phytin contents will be reduced to nought in flours with an ash content about 0.30 g/100 g, or the lowest ash content practically obtainable, flours derived from the inner part of the wheat kernel. The total phosphorus in such flours averages 0.065 g/100 g, which is of the same dimension as the "non-phytin P" in most of the flours examined. This part of the total P is supposed to be bound in proteins, lipoids and in the amylopectin of the starch. In some whole meal types the non-phytin P is a little higher, but greater deviations indicate that the flour is abnormal, for instance damaged by germination.

The phytin P is accumulated in the outer layers of the grain, bran and germ, and it will increase both absolutely and as percent of the total with increasing degree of extraction.

The phytase activity is far greater in rye flours than in wheat and barley. No activity was found in oat meal. The decomposition is very retarded at lower temperatures. In suspensions, the phosphorus in solution consists of inorganic phosphates from phytin decomposition, dissolved phytin, and small amounts of phosphorus from non-phytin compounds, going slowly into solution. A little rest of the total phosphorus is always left undissolved, least by sour reaction. In oat meal, this insoluble phosphorus makes a greater part of the total than in other flours. Part of this is brought into solution in mixtures of rye and oat meal, probably due to some enzymatic process. The phytase of rye whole meal is so powerful that it can split the phytin of oat meal in suspensions completely in a short time, even if the rye makes but 25 % of the flour mixture.

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Release of Contraction and Changes in Birefringence Caused by Adenosine Triphosphate in Isolated Cross Striated Muscle Fibres.[1]

By

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Modern contraction theories are mainly based on the investigation of minute structural properties of myosin and skeletal muscle. Birefringence, X-ray patterns and thermo-elastic properties permit the conclusion that anisodiametric protein chains are the contractile elements (ASTBURY 1939, MEYER and PICKEN 1937, MURALT and EDSALL 1930, WEBER 1934), while the analysis of their mechanical properties indicates the presence of angular movements in these minute structural elements (BUCHTHAL 1942). Our knowledge concerning the transfer of energy from the chemical processes involved in contraction to the contractile elements is rather hypothetical.

A promising attempt to fill this apparently irremediable gap was made by ENGELHARDT and LJUBIMOVA (1939), who found that myosin is an essential link in the enzymatic breakdown of adenosine triphosphate and acts as an adenosine triphosphatase. This interaction is attended by reversible changes in the viscosity and flow birefringence of myosin solutions (NEEDHAM et al. 1941, 1942). It is tempting to correlate these observations with the contraction process in the intact muscle fibre, which is likewise accompanied by changes in birefringence. The interdependence of

[1] A preliminary account of this work was sent for publication to "*Nature*" on March 20th, 1944.

[2] Working with a grant from the *Rockefeller Foundation*.

changes in birefringence and contraction is, however, not absolute, as reversible changes in birefringence are brought about, in the absence of contraction in the isolated excitable muscle fibre, by variation of the hydrogen ion concentration or the salt content in the surrounding medium (BUCHTHAL and KNAPPEIS 1938).

In the present investigation it was, therefore, intended to study how far the results of model experiments, especially those concerning birefringence, are related to the physiological processes and properties of the living muscle fibre. Furthermore, the striking effect of adenosine triphosphate as a chemical stimulus, which was observed in the course of this investigation, was made the object of further analysis.

Method.

The experiments were performed on small muscle bundles (10—20 fibres) and isolated fibres of m. semitendinosus (Rana esculenta and Rana temporaria). During the preparation and the experiments the fibre was kept in a Ringer solution containing 6.7 g NaCl, 0.2 g KCl, 0.14 g anhydrous $CaCl_2$, 0.2 g glucose per litre and a suitable amount of gum arabic buffered with $NaHCO_3$ to pH 7.3. Constant pH and oxygen content were obtained by passing a gas mixture of 1 per cent CO_2 and 99 per cent O_2 through the solution. Its temperature was 12—15° C.

For microscopic observation the isolated fibre or the muscle bundle was placed on a slide and the tendon ends fixed by means of two movable metal clamps. A celluloid frame 0.5 mm thick was fastened upon the slide and could be closed with a cover glass to prevent drying out of the preparation after application of the different substances. The effect of the chemical stimuli was investigated partly by applying them with a fine pipette to the fibre bundle (amounts of liquid 0.03 ml), partly by application to the isolated fibre by means of a micro-pipette in connection with a micro-manipulator (amounts of liquid $4—7 \times 10^3\ \mu^3$ (BUCHTHAL and LINDHARD 1942). Although the nerve ending in frog muscle is spread over a large area of the fibre, end plate-free regions could be investigated. Furthermore, in all cases the chemical stimuli were administered to curarised and non-curarised fibres; complete curarisation was ensured by previously controlling the disappearance of indirect excitability.

Excitability was controlled by single current pulses or tetanic stimuli of short duration from a Harvard coil or a condenser arrangement applied over the metal clamps to the tendon ends of the fibre.

Mechanical tension was registered by means of a condenser-myograph previously described (BUCHTHAL et al. 1944). *Action potentials* were led off by Ag-AgCl electrodes and recorded by a cathode ray oscillograph with suitable A.C. amplification. For the determination of *birefringence* $\left(\frac{\Gamma}{d}\right)$ a polarization microscope was used, as described in

detail in a former paper (BUCHTHAL and KNAPPEIS 1938). The phase difference (Γ) was measured with a BABINET compensator and the fibre thickness (d) by means of an eye-piece micrometer with movable cobweb. White light with a maximum at 550 mμ was used. Experiments were performed on isolated fibres with different degrees of stretch; the latter was determined by measuring the height of compartment (anisotropic (A) plus isotropic (I) substance) on *microphotographs*. Furthermore, in a series of experiments the lengths of A and I were microphotographically determined 0.5—10 minutes after application of the chemical stimulus. The microphotographs were measured on the negative under the microscope, the total magnification amounting to 1000 ×.

Preparation of Substances.[1]

1. *Adenosine triphosphate (ATP).*

The ATP was prepared as the Ba salt by the method described by D. M. NEEDHAM (1942) and purified several times over the Hg and Ba salts. The pyrophosphate P (7′ P) was determined by hydrolysing at 100° C for 7 min. in n. HCl. All phosphate estimations were carried out according to the method of FISKE and SUBBAROW (1925) in the modification of SCHEEL (1936). The analysis of a typical specimen gave: total P, 10.4 %; 7′ P, 6.9 %; N, 7.9 %; calc. for $C_{10}H_{12}O_{13}N_5P_3Ba_2$, $6H_2O$: total P, 10.5 %; 7′ P, 7.0 %; N, 7.9 %. The purity of the substances thus prepared was at least 98—99 %.

A solution of the free acid was prepared by converting the analysed Ba salt into the Hg salt, washing the latter several times with 0.2 n. HNO_3, then with distilled water until neutral to litmus after which the Hg was precipitated with H_2S from the water suspension of the Hg salt. By titrating the solution with n. NaOH to ph 7.3 a solution of the Na salt was obtained. Na ATP solutions containing 16—50 mg ATP (calc. as free acid) were used as staple solutions. The analysis of a typical specimen gave total P, 4.54 mg/ml, 7′ P 2.98 mg/ml; N, 0.34 % corresponding to a content of 24.7 mg ATP/ml (calc. as free acid.). Control analyses showed that such solutions were stable at 15° C for several weeks.

An *iso-osmotic* ATP-Ringer solution was prepared by replacing part of the NaCl + water by an equivalent amount of the staple Na ATP solution. The solutions of the other compounds tested were likewise applied in constant ionic strength by substituting a suitable amount of NaCl in the Ringer.

In control experiments *cozymase* was tested as a possible impurity in our ATP preparations. The cozymase which was kindly put at our disposal by Prof. J. RUNNSTRÖM, had a purity of 60 %.

2. *Adenosine diphosphate (ADP).*

The substance was prepared in the form of the Ba salt according to BAILEY (1942) from pure Ba ATP. Analysis gave: Total P, 8.9; 7′ Ps 4.4; calc. for $C_{10}H_{12}O_{10}N_5P_2Ba_{1.5}$, $4H_2O$; total P, 8.8 %; 7′ P, 4.4.

[1] I. and A. MUNCH PETERSEN assisted in the preparation and the analysis of the substances used.

The staple solution of Na ADP, prepared over the Hg salt as described above under ATP (pH 7.3), gave the following analysis: total P, 1.53 mg/ml; 7′ P; 0.77 mg/ml; corresponding to an ADP content of 10.5 mg/ml (calc. as free acid).

It seemed of interest to compare the action of ATP with that of its probable primary breakdown products, i. e. an equimolar mixture of ADP + inorganic orthophosphate. The solution used contained 10.5 mg/ml ADP (calc. as free acid) + 4.4 mg Na_2HPO_4, $2H_2O$, (pH = 7.3)

3. *Adenylic acid* was prepared from the pure Ba ATP according to KERR (1941) M. P. 189° C (decomp.). The analysis gave: P, 8.9 %; N, 20.2 %; calc. for $C_{10}H_{14}N_5O_7P$; P, 8.9; N, 20.2. The staple solution (brought to pH 7.3 with NaOH) contained 8.6 mg adenylic acid/ml.

Adenylic acid was further tested in the form of an equimolar mixture with inorganic pyrophosphate. The staple solution prepared from adenylic acid and $Na_4P_2O_7$, $10H_2O$ and adjusted to ph 7.3 by addition of n. HCl contained 3.9 mg adenylic acid + 1.9 mg pyrophosphate (calc. as $H_4P_2O_7$)/ml.

4. *Sodium triphosphate* ($Na_5P_3O_{10}$, $5H_2O$).

The substance used contained c. 85 % $Na_5P_3O_{10}$, $5H_2O$ and 12 % sodium orthophosphate. The staple solution was adjusted with n. HCl to pH 7.3 and was sufficiently stable at this pH for our purposes (cf. HUBER 1936).

5. *Sodium pyrophosphate* $Na_4P_2O_7$, $10H_2O$ (pro analysi, MERCK), solution adjusted to pH 7.3 with n. HCl.

6. *Sodium orthophosphate* Na_2HPO_4, $2H_2O$ (SÖRENSEN), solution adjusted to pH 7.3 with n. HCl.

Results.

1. *Effect of adenosine triphosphate (ATP).*

When an iso-osmotic solution of Ringer Na ATP is added to a muscle bundle containing ATP in amounts of 0.05—0.1 mg ($3.6—7.3 \times 10^{-6}$ mol/ml) a short, rapid tetanus-like contraction is initiated, which is followed after a short time by recurrent single twitches continuing for 1—2 minutes. Washing with Ringer solution stops this activity which reappears after a fresh application of the chemical stimulus. This procedure can be repeated 4—5 times on the same preparation. The recurrent activity has a frequency of 1—2 contractions per sec. It is asynchronous in the individual fibres of the bundle and the time elapsing between the application of the chemical stimulus and the start of the propagated mechanical response varies considerably in different fibres probably owing to differences in the permeation of the substance. When threshold amounts c. 0.02 mg (1.2×10^{-6} mol/ml) are applied, no recurrent activity occurs, the fibre only reacting with a short tetanus-like contraction. The effect of ATP is identical in the curarised and non-curarised preparations.

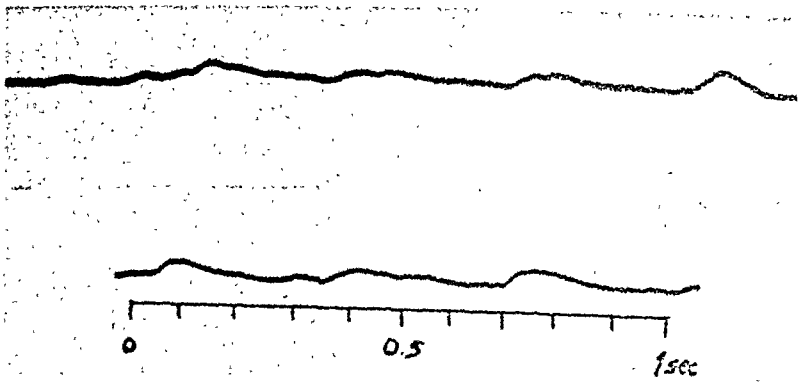
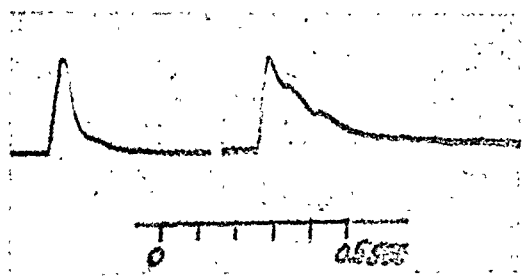


Fig. 1. Mechanical response of a small muscle bundle after application of 0.1 mg adenosine triphosphate (7.3×10^{-6} mol/ml).

The *mechanical response* of a small muscle bundle recorded with a condenser-myograph after application of ATP amounts to about 5—15 per cent of the tension initiated by a single electric stimulus, when all fibres act synchronously (Fig. 1). The effect of electric stimulation before and after application of the chemical stimulus is essentially different (Fig. 2). The mechanical response lasts considerably longer when the fibre has previously been treated with ATP, an effect which is probably due to the release of recurrent activity of some fibres in the bundle by the electric stimulus. Apparently ATP provides a tendency to rhythmic activity, and a fibre previously treated with ATP responds with more than one reaction when a single electric stimulus is applied.



a b

Fig. 2.

a. Mechanical response of a muscle bundle following a single electric stimulus *before* application of adenosine triphosphate.

b. *after* application of 0.1 mg adenosine triphosphate (7.3×10^{-6} mol/ml).

The repetitive activity is furthermore evident from *action potentials* recorded after application of the chemical stimulus. Fig. 3 shows action potentials from bundles with few fibres only after treatment with 0.05 mg (a) and from another bundle after application of 0.1 mg ATP (b_{1-3}). The first large deflection is due to the electric charge of the added drops and is likewise present in the control experiment (b_4) after addition of Ringer solution.

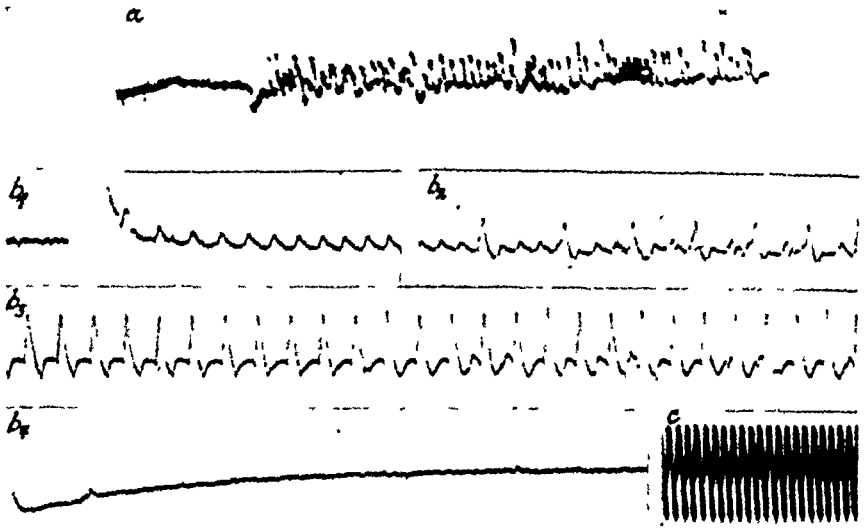


Fig. 3.

a. Action potentials from a muscle bundle (c. 10 fibres) after application of 0.05 mg adenosine triphosphate (3.6×10^{-6} mol/ml).
b_1. action potentials from a muscle bundle (c. 5 fibres) after application of 0.1 mg adenosine triphosphate (7.3×10^{-6} mol/ml).
b_2. 3 sec after application to (b_1).
b_3. 18 sec after application to (b_1). Total duration of activity c. 37 sec.
b_4. control with application of a drop of Ringer solution.
c. calibration 0.1 mV, frequency (time marks) 10 cycles per sec.

In order to determine the smallest amount of ATP producing a mechanical reaction the substance is applied to isolated curarised and non-curarised fibres by means of a micro-pipette with an opening of c. 20 μ diameter. *The threshold dose of adenosine triphosphate directly applied to the fibre is* 1.3—1.7 $\times 10^{-5}$ μg and produces a local contraction. On account of the time necessary for permeation the effective quantity reaching the fibre substance is probably much less. Propagated responses with subsequent repetitive activity are released by approximately a double amount of the substance. No differences are found between the threshold of the non-curarised and the curarised fibres for the chemical stimulus, while the electrical excitability reveals the well known differences.

Birefringence decreases by about 20—30 per cent during tetanic contraction of a single fibre (BUCHTHAL and KNAPPEIS 1938). After a number of contractions a reversible decrease of birefringence occurs, the degree of which depends upon the number of contractions performed. *After application of ATP birefringence*

likewise decreases and attains its minimum about 2 minutes later than after electric stimulation. The subsequent increase corresponds closely to that after electric stimulation and is completely reversible. In Fig. 4 a comparison is given between the course of birefringence after 5 sec of electric stimulation and after the application of 0.37 mg ATP (3.6×10^{-6} mol/ml). In both cases the fall in birefringence amounts to approximately 20 per cent and the following spontaneous increase has a linear course.

In a fibre which is poisoned with *mono-iodoacetic acid* and stimulated to complete fatigue, birefringence does not decrease (BUCHTHAL and KNAPPEIS 1938). It likewise remains constant in the poisoned fibre after an electrically induced tetanic contraction of 5 sec duration, which otherwise causes a fall of about 20 per cent. The curves in Fig. 5 show the effect of ATP on fibres

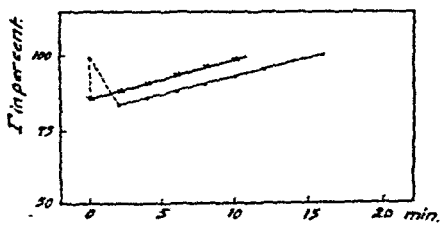


Fig. 4. Phase difference (Γ) as a function of time after tetanic electrical stimulation of 5 sec (×—×—×) and after application of 3.6×10^{-6} mol/ml adenosine triphosphate (●—●—●).
Ordinate: Γ in per cent.
Abscissa: time in minutes.

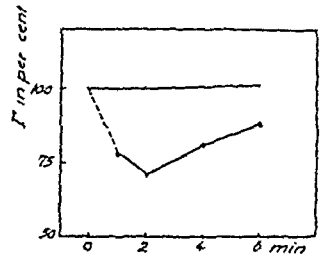


Fig. 5. Phase difference (Γ) in a normal fibre (lower curve) and in a fibre poisoned with iodoacetic acid (1:10,000) after application of 7.3×10^{-6} mol/ml adenosine triphosphate (upper curve).
Ordinate: Γ in per cent.
Abscissa: time in minutes.

of the same muscle with and without previous application of iodoacetate (1 : 10 000). After poisoning, the effect of ATP on birefringence, like that of electrical stimulation, is abolished.

Repeated controls ensure that the *fibre thickness* is constant throughout the experiment and unaltered by ATP or the other substances applied in an iso-osmotic state in this investigation. Changes in birefringence, therefore, are characterised by the variations in phase difference (Γ) in per cent of its original value.

With *increasing concentration* of ATP, both the fall in birefringence and its period of recovery increase (Fig. 6). The minimum in phase difference occurs, however, independent of the concentration approximately 2 minutes after application of the chemical stimulus. The threshold concentration causing a decrease of 5—10 per cent in phase difference is $1.4—1.8 \times 10^{-6}$ mol/ml.

In order to investigate whether the persisting change in birefringence is caused by the occurrence of a contracture-like contraction or is due to after-effects which are independent of the mechanical response and the changes in cross striation accompanying the latter, *micro-photographs* of the fibre were taken in a number of experiments from 25 sec to 10 min after application of ATP. A contraction, both a twitch or a persisting mechanical response, is accompanied by considerable changes in the proportion between the lengths of the anisotropic (A) and isotropic (I) substances. At the maximum of contraction the ratio $\frac{A}{A+I}$ has fallen from 0.62 to 0.53. When the first micro-photograph is taken 25 sec after application of the chemical stimulus, changes in cross striation are already restored in spite of the continuously decreasing phase difference. The changes in birefringence following application of ATP, therefore, are not due to a contracture-like condition of the muscle fibre, *but are the expression of restitutional processes in muscle proteins.* (Fig. 7.)

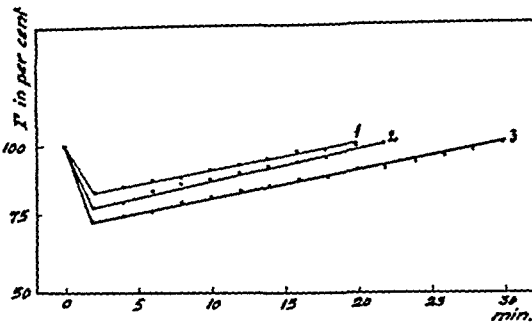


Fig. 6. Application of different concentrations of adenosine triphosphate to the isolated muscle fibre.

Curve 3: 4.9×10^{-6} mol/ml.
Curve 2: 3.6×10^{-6} mol/ml.
Curve 1: 2.5×10^{-6} mol/ml.

Ordinate: Γ in per cent.
Abscissa: time in minutes.

Application of ATP to *electrically unexcitable* fibres results in changes in birefringence similar to those occurring in the excitable fibre, though no contraction is released by the addition of the chemical stimulus. These observations likewise indicate the independence of changes in birefringence and contraction.

Apart from the concentration of the chemical stimulus, the variation in birefringence depends on the *degree of stretch of the muscle fibre.* The fall in phase difference has a maximum at equilibrium length (length 100, c. 36 per cent) and decreases linearly with increasing elongation. At length 175 birefringence only decreases by c. 18 per cent. The curve in Fig. 8 is interpolated

mol/ml. The substance is without effect both on the mechanical response and on the birefringence.

10. *Adenosine diphosphate* (*ADP*).

Application of ADP in concentrations of 0.04—0.1 mg (2.9—8.7 $\times 10^{-6}$ mol/ml) to a small bundle like that of adenosine triphosphate results in the release of a short, tetanus-like contraction with subsequent twitch-like repetitive activity. The threshold dose with micro-application to the isolated fibre is 2—2.5 $\times 10^{-5}$ μg and produces a local response. Propagated contractions and recurrent activity are released by considerably higher amounts (5—10 times the threshold value). The threshold amounts and the durations of activity are the same in the curarised and non-curarised fibres. The *birefringence* shows a fall and recovery period after application of ADP similar to that described for ATP (Fig. 9).

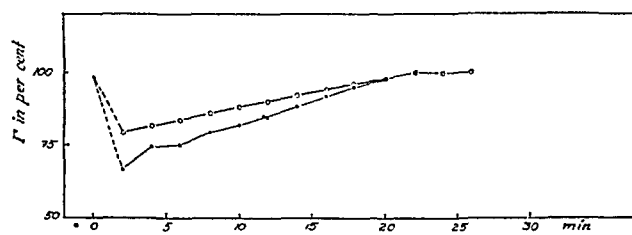


Fig. 9. Phase-difference (Γ) as a function of time after application of 7.3 $\times 10^{-6}$ mol/ml adenosine diphosphate (●—●—●), 8.7 + 8.7 $\times 10^{-6}$ mol adenosine disphosphate plus orthophosphate (○—○—○).

Ordinate: Γ in per cent.
Abscissa: time in minutes.

3. *Adenosine diphosphate plus orthophosphate* likewise initiates contraction, the threshold amount when applied to a small muscle bundle being 0.07 + 0.03 mg (5.2 + 5.2 $\times 10^{-6}$ mol/ml). When administered to the isolated fibre local responses are released by c. 2.0 + 0.85 $\times 10^{-5}$ μg. It is rather striking that the decrease in birefringence is always considerably less (c. 10 per cent) than that produced by corresponding molar amounts of ATP or ADP (Fig. 9). No differences are found between the reactions of curarised or non-curarised fibres.

4. *Adenylic acid* in molar concentrations that correspond to highly effective doses of ATP neither release contraction nor affect birefringence (Fig. 10).

5. *Adenylic acid plus pyrophosphate* release a mechanical response of the same type as that initiated by ADP. Threshold amounts, when applied to a small muscle bundle, are 0.075 +

0.035 mg ($7.2 + 7.2 \times 10^{-6}$ mol/ml), a molar concentration which corresponds to that examined in the case of ATP. Birefringence decreases by 20—30 per cent and, in contrast to the effects of ATP and ADP, *already reaches minimum values after 1 minute* (Fig. 10). The subsequent rise to the original values takes 15—20 minutes.

Apart from these organic phosphates the following related *inorganic phosphorous compounds* were tested:

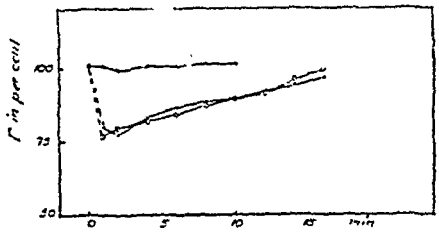


Fig. 10. Phase difference (Γ) as a function of time after application of adenylic acid (7.2×10^{-6} mol/ml) (×—×—×), inorganic pyrophosphate 2.7×10^{-6} mol/ml (○—○—○) and adenylic acid plus pyrophosphate $7.2 + 7.2 \times 10^{-6}$ mol/ml (●—●—●). The concentration of pyrophosphate is equal to that of the preceding curve.

Ordinate: Γ in per cent.
Abscissa: time in minutes.

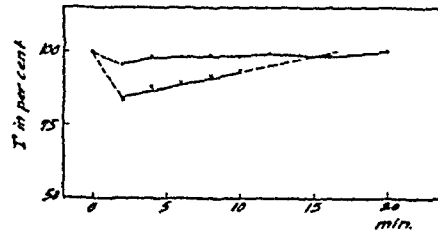


Fig. 11. Phase difference (Γ) as a function of time after application of 6.1×10^{-6} mol/ml sodium triphosphate (●—●—●). Effect of 7.3×10^{-6} mol/ml adenosine triphosphate after previous treatment with 6.1×10^{-6} mol/ml sodium triphosphate (×—×—×).

Ordinate: Γ in per cent.
Abscissa: time in minutes.

Sodium tri-polyphosphate initiates mechanical responses in curarised and non-curarised fibre bundles. The threshold amounts are approximately 0.07 mg (5×10^{-6} mol/ml). Slightly higher concentrations (0.09 mg = 6.1×10^{-6} mol/ml) release long-lasting, repetitive twitch-like activity which is asynchronous in the different fibres of the bundle. Micro-application to the isolated fibre has a threshold of 2.0×10^{-5} μg and produces local contractions. With concentrations acting as highly effective chemical stimuli to the fibre the fall in birefringence is either absent or slight (Fig. 11). When ATP is added to the fibre after previous treatment with inorganic triphosphate its effect on birefringence is considerably reduced, while otherwise repeated application of ATP causes equal decreases in Γ.

Sodium pyrophosphate when applied to a curarised or non-curarised muscle bundle causes persistent *contracture-like* responses in threshold amounts of 0.2 mg (14.8×10^{-6} mol/ml). Recurrent, twitch-like activity does not occur even if the substance is applied in higher concentrations. The threshold for

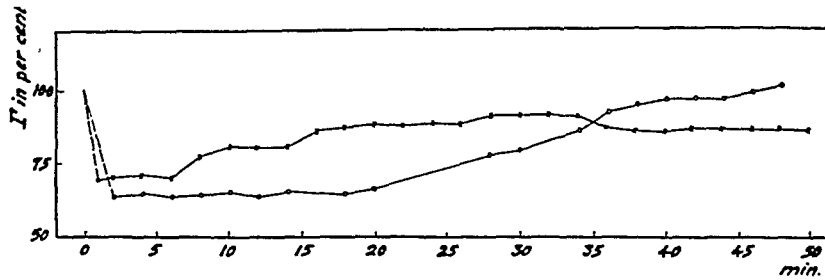


Fig. 12. Phase difference (Γ) after application of 4.9×10^{-6} mol/ml (○—○—○) and 9.9×10^{-6} mol/ml (×—×—×) sodium pyrophosphate.

Ordinate: Γ in per cent.
Abscissa: time in minutes.

micro-application is c. 4.8×10^{-5} μg and there also the local mechanical response has a considerably longer duration than after addition of sodium triphosphate.

The threshold amount producing a fall in birefringence is lower ($< 0.45 \times 10^{-6}$ mol/ml) than the lowest concentrations stimulating the fibre (4.8×10^{-6} mol/ml). Threshold concentrations for the mechanical response cause irreversible changes in birefringence (Fig. 12). The highest concentration at which the phase difference still returns to its original value is (4.9×10^{-6} mol/ml). As is the case with adenylic acid plus pyrophosphate, the fall in birefringence reaches its maximum after 1 minute when pyrophosphate is applied (Fig. 13). The different rates in the decrease of Γ are probably due to the differences in the permeation of the substances applied.

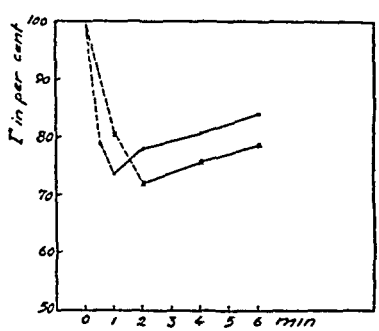


Fig. 13. Initial course of phase difference (Γ) after application of 7.4×10^{-6} mol/ml sodium pyrophosphate (●—●—●) and 7.3×10^{-6} mol/ml adenosine triphosphate (×—×—×).

Ordinate: Γ in per cent.
Abscissa: time in minutes.

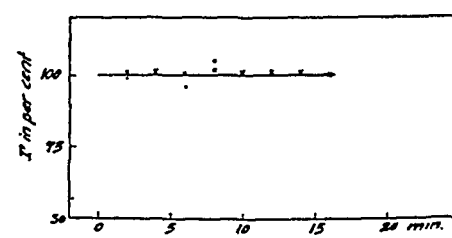


Fig. 14. Phase difference (Γ) after application of 12.5×10^{-6} mol/ml (×—×—×) and 24.5×10^{-6} mol/ml (●—●—●) sodium orthophosphate.

Ordinate: Γ in per cent.
Abscissa: time in minutes.

Inorganic sodium orthophosphate acts rather inconstantly as a chemical stimulus to curarised and non-curarised fibres. The threshold dose varies and even amounts of 0.13 mg release a mechanical contracture-like response only in 8 of 19 preparations. Once treated, the fibre is insensitive to a further dose of orthophosphate, even when it is washed with Ringer solution. The birefringence remains unchanged by inorganic orthophosphate in amounts of ($12.4 - 24.7 \times 10^{-6}$ mol/ml). It may be mentioned that *orthophosphate* shows an *inhibitory effect* on subsequent doses of ATP.

Discussion.

Two mutually independent observations are the main result of this investigation: viz. the effects of adenosine triphosphate, adenosine diphosphate and inorganic triphosphate in the curarised and non-curarised fibres as chemical stimuli initiating twitch-like and short tetanus-like contractions, and the long-lasting and reversible changes in birefringence caused by some of these phosphorous compounds. Both findings establish that ATP permeates the "membrane" of the muscle fibre, a fact which is apparently in disagreement with the statements of BOYLE and CONWAY (1941).

Although muscle can be induced to contract by the application of almost any agent which, if applied in a more intense form, would injure or destroy it, the polyphosphates investigated have a specific effect since they act in extremely small doses. It seems therefore justified to reckon with their physiological importance. Experiments by ENGELHARDT and LJUBIMOVA (1939), NEEDHAM et al. (1941, 1942), and SZENT GYÖRGYI et al. (1941, 1942) indicate a close connection between ATP and the protein molecule, although it must be born in mind that these observations do not prove that the breakdown of ATP is directly associated with contraction in the *living* muscle fibre. However, the correlation between myosin and ATP on the one hand, and the extreme sensitivity of muscle substance to ATP on the other, support the suggestion made by NEEDHAM on the basis of the mentioned model experiments that ATP is the normal agent of contraction. The effect of a stimulus would then be to establish contact between the enzyme (myosin) and the energy-supplying substrate (ATP). Addition of ATP would thus correspond to the establishment of the contraction which is otherwise brought about by the impulse from the motor end plate. If this view is correct, ATP normally present in

resting muscle cannot be directly in contact with the contractile micellae, and we must either suppose differences in local distribution as CASPERSSON and THORELL'S (1942) finding may indicate or the presence of a non-reactive compound.

The other observation is the change in birefringence brought about by the above substances.

It must be pointed out, however, that the changes in birefringence registered in this investigation are persistent after-effects and reach their maximum a considerable time after the mechanical response has come to an end, i. e. they are restitution phenomena. Like NEEDHAM et al. for the flow birefringence of myosin, we find that birefringence of muscle is hardly affected by inorganic triphosphate. NEEDHAM et al. conclude that the purine-ribose end of the ATP molecule should be of great importance in changing the shape of the protein molecule. The stimulating effect shown by triphosphate in spite of the unchanged birefringence indicates that the changes in the protein molecules expressed in birefringence may be other than those accompanying contraction. The mutual independence of molecular changes manifested in birefringence and those due to contraction is furthermore emphasised by the fact that the action of ATP on birefringence is abolished when the fibre is treated with iodoacetate, in spite of retained excitability by ATP and other stimuli.

The changes in muscle protein represented by changes in birefringence and those associated with the release of contraction are both initiated by ATP.

Thus, the enzymatic breakdown of ATP, the chemical reaction nearest in time to the contraction proper, but still considered to belong to restitution processes, probably occurs even at the primary physical stage of contraction.

There is evidence of a different kind that the minute structural elements in muscle are the site of potential energy, the chemical processes involved in muscular activity serving to recharge the system discharged during contraction (BUCHTHAL and LINDHARD 1939). It is tempting to assume that ATP is the agent that transfers the chemical energy generated in muscle to its contractile elements both in the discharge and the recharge of the muscle machine. This would mean that ATP, apart from the energy-rich phosphate bond (LIPMANN 1941), possesses a *specific* molecular structure adapted to direct interaction with the contractile elements.

The results obtained with ADP and ADP + orthophosphate on the one hand and inorganic triphosphate on the other imply that both the triphosphate and the nucleotide part of the ATP molecule are in themselves energetically and structurally sufficient to release contraction, but only the nucleotide part seems adapted to be recharged for the transfer of energy from other energy supplying processes. Taking the changes in birefringence as an expression of restitution, i. e. protein recharge, leads to the conclusion that iodoacetate inhibits one or several processes essential in this restitution. The assumption of ATP as an agent of contraction and energy recharge agrees well with the establishment of myosin as an adenosine triphosphatase.

Summary.

1. Micro-application of adenosine triphosphate (a 99 per cent preparation) in iso-osmotic Ringer solution releases in threshold amounts of $1—2 \times 10^{-5}$ μg a brief tetanus-like activity in non-curarised and curarised isolated striated muscle fibres.

2. A mechanical response following electric stimulation lasts considerably longer when a small fibre bundle has previously been treated with adenosine triphosphate than it does before application of the substance.

3. Application of adenosine triphosphate causes a reversible fall in birefringence. This decrease is abolished by poisoning with iodoacetate.

4. The fall in birefringence amounts to 10—30 per cent, increasing with increasing concentration of adenosine triphosphate, and decreases with increasing stretch of the fibre.

5. While the initial fall in birefringence following electrical stimulation occurs more rapidly than after application of adenosine triphosphate, the time of restitution is 10—20 min, being identical in both cases. The fibre has regained its resting condition, when birefringence reaches its minimum, and the protracted return is regarded as an expression of restitutional processes in muscle proteins.

6. Adenosine diphosphate and adenosine diphosphate + orthophosphate act like adenosine triphosphate both as regards initiation of contraction and effect on birefringence. In the case of adenosine diphosphate + orthophosphate the decrease in bire-

fringence is, however, essentially less than after application of adenosine triphosphate and adenosine diphosphate.

7. Adenylic acid has no effect on birefringence or contraction. Adenylic acid + pyrophosphate release contraction and cause a decrease of birefringence which reaches its minimum more rapidly than after application of adenosine diphosphate.

8. Inorganic sodium triphosphate releases contraction, while its influence on birefringence is absent or slight.

9. Sodium pyrophosphate causes contracture-like mechanical responses. Threshold amounts for the release of contraction are accompanied by irreversible changes in birefringence.

10. Sodium orthophosphate has no influence on birefringence and acts, even in large concentrations inconstantly as a chemical stimulus; the response, if present, is protracted. Birefringence remains unchanged.

11. The energy-rich phosphate bond in the form of adenosine-triphosphate is not only the immediate source of energy recharge, but also the agent for the release of contraction.

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A Method of Driving the Brodie-Palmer Time-Marking Clock from the A. C. Mains.

By

A. G. HILDING BJURSTEDT.

Received 31 August 1944.

The Brodie-Palmer time-marking clock, so generally used in physiological laboratories, ordinarily requires frequent recharging of the 4-volt storage battery supply. To maintain the pendulum swing and to work the recording signal impulses of a current are needed, too strong to be drawn from the D. C. mains by appropriate resistors in series with the electromagnetic coils. Each time the circuits are broken arcs ensue, soon resulting in destroyed contacts. The inconvenience of battery supply may, however, be removed by operating the pendulum and signal on alternating current. Thus, the A. C. mains can be used as power supply without altering the set wiring.

A small transformer is needed, by which the line voltage is lowered to 25 volts. When using only one signal the maximum current required from the secondary is 3 ampères. The secondary is connected to the terminals marked »Battery». In series with the signal, which is connected to the »Signal» terminals, a resistor of 5 to 10 ohms is inserted to reduce the voltage fall in the signal coils to about 6 volts. The intermittent magnetization of the pendulum and signal coils has no practical drawback.

Additional signals may be connected to the »Signal» terminals by using a transformer with a secondary current permitting an increased load of 2 extra ampères per additional signal and by inserting a resistor in series with each signal.

The A. C. powered set has proved very convenient and stable in operation. The average power consumption amounts to about 5 watts.

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Histamine as a Physiological Excitant of Acid Gastric Secretion.

By

NILS EMMELIN and G. S. KAHLSON.

Received 6 July 1944.

Two conceptions as to the rôle of histamine in gastric secretion have been put forward. The fact that histamine is the sole gastric secretory excitant in dilute acid extracts of the pyloric mucosa was taken to indicate that histamine might be identical with "gastrin", the hormone instrumental in the gastric phase of secretion. As stressed by SACKS et al. (1932) the crucial question to be answered in this connection is whether the histamine concentration of the blood plasma rises during the gastric phase. MACINTOSH (1938), using the method of BARSOUM and GADDUM, found that the histamine concentration of the systemic blood was not significantly affected by the digestion of a meal. As discussed by MACINTOSH, there may have occurred in his experiments an increase in the histamine content of the plasma, which could not be detected in experiments with whole blood, and which gives values representing mainly the histamine content of the corpuscles. Experiments during the gastric phase with plasma, which normally contains very small amounts of histamine as compared with the corpuscles, have not been published, as far as we know. The questions in regard to the rôle of histamine as a blood-born agent active in the gastric phase have lost much of their bearing by the recent work of KOMAROV (1941) and UVNÄS et al. (MUNCH-PETERSEN, RÖNNOW and UVNÄS 1944); these authors have isolated from the pyloric mucosa a protein-like substance free from histamine which on intravenous injection causes a profound secretion from the fundic glands.

BABKIN and MACINTOSH suggested that histamine might play a part in the cephalic phase of gastric secretion by serving as a chemical mediator of vagal impulses to the parietal cells. This hypothesis is supported by the facts that in the dog the fundic mucosa contains more histamine than the pyloric (GAVIN et al. 1933) and that the gastric juice, according to some workers (BROWN and SMITH 1935; KOMAROV 1936), contains a histamine-like substance, which is denied by BLOCH and NECHELES (1938). MACINTOSH (1938) put this hypothesis to the test. In experiments on dogs he showed that the histamine content of gastric juice collected during the cephalic phase was higher than in blood plasma. MACINTOSH discusses the possibility that during the cephalic phase histamine is liberated within or near to the parietal cells as a result of stimulation of the vagus, thus "acting in the first phase of gastric secretion as a local hormone, rather than in the second phase as a true hormone".

It was shown in this laboratory that the vagi control parietal secretion by the mediation of a histamine-free pyloric agent which is liberated by vagal impulses and carried by the blood stream to the parietal cells (UVNÄS 1942). We have investigated whether this pyloric agent, which is probably identical with the hormone engaged in the gastric phase, liberates histamine from the stores in the fundic mucosa. In the course of these experiments we have studied the liberation of histamine during gastric secretion induced by many different modes of stimuli. It is assumed that if histamine is liberated within the mucosa, this substance partly enters the parietal cells, emerges in the gastric juice and partly escapes into the blood stream. Our experiments with blood plasma are still proceeding, while the results with gastric juice are presented here. — Since many of our experiments were performed on cats, we have incidentally supplemented the work of GAVIN, MCHENRY and WILSON (1933) by determining the histamine content of the cat's gastric mucous membrane.

A. Methods of Assay and Identification of Histamine.

Fundic and pyloric mucous membrane was obtained from cats killed by bleeding whose blood was used for other purposes. The dogs, before dissecting the mucous membrane, were used for experiments on reactive hyperaemia, and were killed by air embolism. The mucous tissue was minced by scissors, ground with sand in a mortar, and

extracted by CODE's (1937) modification of the BARSOUM-GADDUM method (1935) as used in this laboratory (EMMELIN et al. 1941). The blood plasma was also treated by these methods. The gastric juice was filtered, neutralized, and tested as described below. In some instances the gastric juice, previous to the biological assay, was extracted by CODE's method. All figures obtained with extracted material are given in brackets.

The assay was performed by these methods:

a) The guinea-pig's ileum, suspended in Tyrode's solution containing atropine sulphate 1 : 200,000; this was used as a routine method.

b) The blood pressure of the cat anaesthetized by chloralose.

c) The smooth muscles of the guinea-pig's and cat's bronchi which constrict when histamine is injected intravenously (EMMELIN et al. 1941).

d) The motor effect on the cats small intestine as indicated by a balloon introduced in the gut.

e) The stimulating effect on the cat's acid gastric secretion.

When using the last three methods the gastric juice was concentrated. This was not necessary in the more sensitive methods a) and b).

All figures in this paper are calculated in terms of histamine base.

Since none of these five methods is specific for histamine, the substance assayed was identified by the following means and considerations:

1) The agent, in this paper referred to as histamine, was inactivated by incubation with histaminase. We used a very active and highly purified specimen of histaminase prepared by SWEDIN (1943).

2) The histamine-like activity on the gut disappeared after treatment with thymoxyethyldiethylamine[1] (BOVET and STAUB 1937, STAUB 1939, ROSENTHAL and MINARD 1939) or theophyllinmonoethanolamine[1] ("Theamine"), on the bronchi and on the cat's blood pressure after injection of this latter substance (EMMELIN et al. 1941).

3) Assays obtained by different biological methods show a reasonable quantitative agreement.

B. The Histamine Content of the Gastric Mucosa.

Table 1 gives the histamine content of the fundic as compared with the pyloric mucosa in three cats and four dogs. The tests were performed on the guinea-pig's gut and bronchi.

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[1] For the supply of these substances our thanks are due to Dr. A. DEUTSCH, head of the Chemical Laboratory of "Leo", Ltd, Hälsingborg, who synthetised them for us.

Table 1.

Histamine content of gastric mucous membrane in the cat and the dog.

	Histamine content, mg/kg	
	fundus	pylorus
Cat 1	5.3	4.3
Cat 2	19.0	6.5
Cat 3	34.0	16.0
Dog 1	55.0	25.0
Dog 2	55.0	30.0
Dog 3	180.0	80.0
Dog 4	48.0	24.0

C. The Histamine Content of the Gastric Juice.

1. *Spontaneously secreted juice.* Cats, decerebrated or under chloralose were used; the vagi were cut. The rate of secretion was very low. In 12 cats acid juice was collected under these conditions. The histamine content of the juice is presented in table 3. Some samples of juice were extracted according to CODE; the corresponding figures are given in brackets. The other samples, after neutralizing, were tested directly on the guineapig's gut. The spontaneously secreted juice is contaminated by varying amounts of mucus and saliva.

Table 2.

Histamine content of spontaneously secreted gastric juice in 12 cats. Figures in brackets refer to extracted samples; the others are tested directly on the gut.

Cat nr.	1	2	3	4	5	6	7	8	9	10	11	12
Histamine content γ per litre	(55)	(6)	(14)	25	(5)	(45)	9	6	45	40	(5)	40

2. *The cephalic phase.*

a) *Sham feeding.* In dogs gastric pouches of the PAVLOV or of the type described by NEUWELT et al. (1940) and oesophageal fistule according to KOMAROV (1926) were prepared. The dogs were fed with palatable meat during 30—60 minutes, and acid juice was collected from the gastric cannula. The juice was tested either directly on the gut after neutralization or by the several

biological tests described previously after extraction by the CODE procedure. In every instance the juice contained histamine. Fig. 1 represents an experiment of this kind. Samples were collected during 3—7 subsequent periods of 15 minutes.

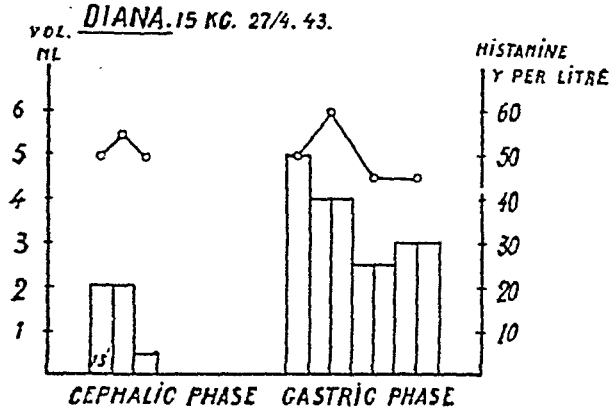


Fig. 1. Histamine content of gastric juice from the cephalic and gastric phases.

In the experiment presented in fig. 2 only one specimen of juice was collected daily during a period of about two months.

In a special series of experiments large amounts of gastric juice were collected during sham feeding, extracted, concentrated, and assayed by several methods. Fig. 3—5 refer to such an experiment. 140 ml juice were treated according to CODE, and concentrated to 6 ml. In the cat 0.1—0.2 ml of this extract intravenously caused a fall in blood pressure which was matched against the effects caused by a standard solution of histamine (fig. 3 A). The fall in blood pressure caused by the extract persisted after atropinization of the cat (fig. 3 B), and disappeared almost entirely when "theamine" had been previously injected (fig. 3 C). — This extract was also injected intravenously in the guinea-pig. The resulting bronchoconstriction was matched against histamine (fig. 4 A); if "theamine" had previously been injected in the guinea-pig, neither the extract nor histamine constrict the bronchi (fig. 4 B). — In fig. 5 the same extract is assayed on the guinea-pig's gut. The activity of the extract is abolished by thymoxyethyldiethylamine. Tested on the cat's blood pressure and on the bronchi the histamine activity of the gastric juice amounts to 35—45 γ per litre and on the gut to 34—38 γ per litre.

b) *Electrical vagus stimulation.* Dogs and cats were anaesthetised by chloralose or decerebrated, the vagi cut and stimulated in the neck or in the chest below the heart with the chest closed

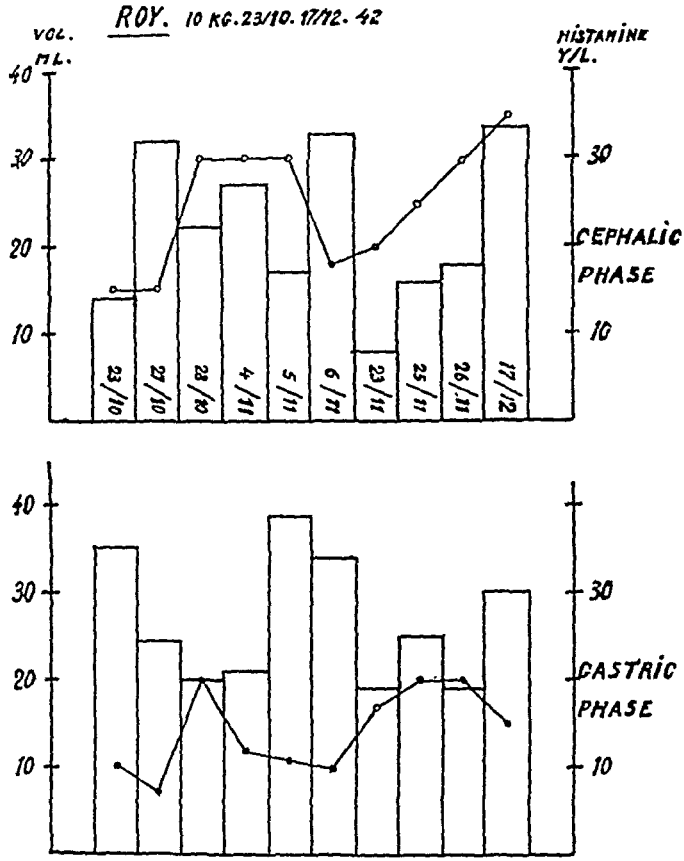


Fig. 2.

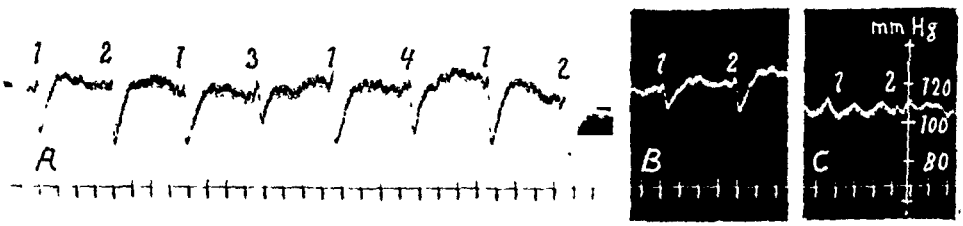
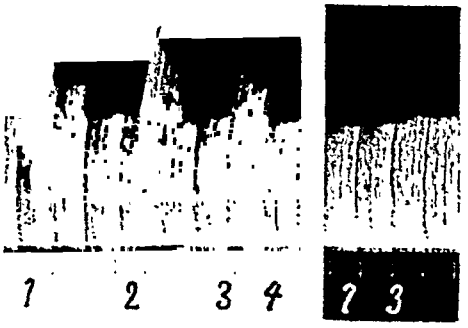


Fig. 3 A. Cat 2.2 kg, chloralose-urethan. Arterial blood pressure, mercury manometer. Intravenous injections every four minutes of extracted gastric juice (0.20 ml at 2, 0.10 ml at 3, 0.15 ml at 4) and of histamine disphosphate 0.20 γ at 1. Time marks in minutes.

Fig. 3 B. Atropine sulphate. 1 mg per kg, was given intravenously. Extract (at 2) and histamine (at 1) are still active.

Fig. 3 C. "Theamine", 25 mg per kg, was injected intravenously. Extract and histamine in the same amounts as in fig. 3 B are almost without effect on the blood pressure.



A B

Fig. 4. Guinea-pig, 620 g anaesthetized with urethane. Bronchoconstriction is indicated by increased upward movements of the lever. Extract (0.5 ml at 1) and histamine (1 γ at 2; 0.5 γ at 3) are injected intravenously. "Theamine", 25 mg per kg is injected at 4, rendering the bronchi insensitive to the motor effect of extract (1) and histamine (3). Time in minutes.

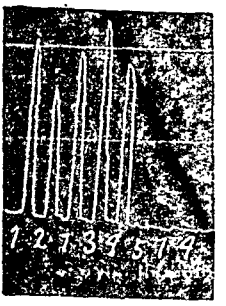


Fig. 5. Guinea-pig's ileum suspended in 2 ml Tyrode's solution containing atropine sulphate 1 : 200,000. Histamine is added at 1 (0.015 γ), extract 1 : 20 at 2 (0.30 ml), at 3 (0.40 ml) and at 4 (0.35 ml). Thymoxyethyldiethylamine (2 γ) is added at 5, rendering the muscle insensitive to histamine (1) and extract (4). Time in minutes.

and spontaneous breathing when the electrodes are applied (WRIGHT et al. 1940). Tables 3 and 4 give the results in cats and dogs. The figures obtained after extraction of the juice

Table 3.

The histamine content of gastric juice obtained on vagal stimulation in 19 cats.

Cat nr	Duration of stimulation, minutes	Volume of gastric juice in minutes	Histamine content γ per litre
1	25	6.5	(50)
2	7	2.5	(150)
3	30	4.0	(4)
4	20	2.5	(90)
5	—	—	16
6	20	5.0	(20)
7	40	3.0	(14)
8	30	11.4	25
9	—	—	50
10	35	3.0	(15)
11	75	4.5	(5)
12	45	6.0	(20)
13	—	—	30
14	80	8.0	(6)
15	75	6.3	45
16	55	7.8	(25)
17	15	4.2	(75)
18	75	12.0	(25)
19	60	12.1	35

Table 4.

The histamine content of gastric juice obtained on vagal stimulation in 7 dogs.

Dog Nr	Duration of stimulation, minutes	Volume of gastric juice in minutes	Histamine content γ per litre
1	15	15	(8)
2	45	14	(11)
3	—	—	40
4	—	—	35
5	30	7	(35)
6	—	—	120
7	60	20	(8)

are given in brackets; these extracts were tested by several biological methods. The native juice was tested on the gut.

c) *Central stimulation by acetylcholine.* In four anaesthetized cats 0.15—0.5 mg acetylcholine was injected in the lateral cerebral ventricle. This procedure was shown by HENDERSON and WILSON (1936) to stimulate gastro-intestinal activity in man. The acid juice secreted after the injection of acetylcholine was tested for its histamine activity (table 5).

Table 5.

The histamine content of gastric juice obtained on injection of acetylcholine in the lateral cerebral ventricle.

Cat nr	Acetylcoline mg	Volume of gastric juice in ml	Histamine content of juice, γ per litre
1	0.50	4.5	25
2	0.20	10.0	25
3	0.20	4.3	45
4	0.15	3.0	40

3. *The gastric phase.* In the dogs referred to in the paragraph on the cephalic phase, meat and watery meat-extracts were introduced in the main stomach by a tube and gastric juice collected from the pouch. The collection of juice was started 30 minutes after feeding. Reference to the results is given in fig. 1 and 2, where the figures from the cephalic and gastric phases are presented together.

In two other dogs with gastric pouches but not fitted with oesophageal fistulae, gastric juice was collected from the pouch during the "gastric" phase of digestion. On direct testing on

the gut the juice was found to contain histamine ranging from 30 to 80 γ per litre.

Large amounts of canine gastric juice from the gastric phase were extracted and concentrated as described in the experiments referred to in figs. 3, 4 and 5. This extract was slowly injected intravenously in cats, and the gastric secretion measured. Table 6 exemplifies an experiment of this kind. 15 ml extract, corresponding to 610 ml gastric juice, was injected intravenously during 10 minutes. The secretory effect exceeds that of 20 γ histamine injected during 10 minutes.

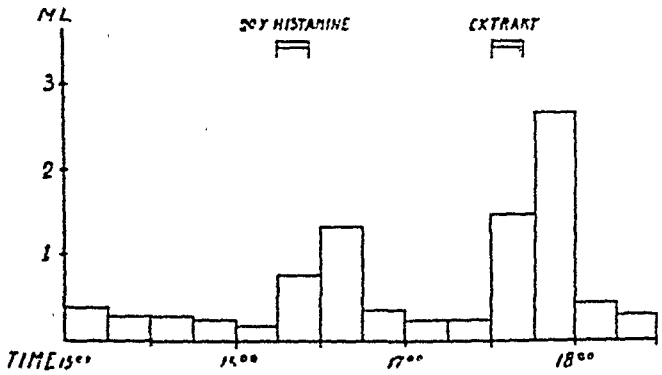


Fig. 6. Concentrated gastric juice injected intravenously in the cat elicits gastric section.

4. *Juice obtained on injection of eserine.* In a cat under chloralose 1 mg eserine salicylate was injected intravenously. The juice secreted during the first and second hours after the injection contained 30 and 35 γ/l respectively. The juice was tested directly on the gut.

5. *Juice obtained after injection of "gastrin".* The gastrin was prepared by the methods deviced by UVNÄS et al. (1944) in this laboratory and injected intravenously in cats under chloralose. The injection of this histamine-free preparation was followed by profound secretion of acid juice. In seven cats the juice collected after injection of "gastrin" contained the following amounts of histamine in γ per litre: 20, 20, 30, 35, 35, 45, 55. The assay was performed with native juice.

6. *Injection of "gastrin" combined with vagus stimulation.* In three cats "gastrin" was injected intravenously, and subsequent to the injection both vagi were stimulated electrically. This procedure was very active in stimulating acid secretion. The three native specimen of juice contained 14, 25 and 45 γ per litre.

7. *Secretion caused by "Priscol"*. It has been shown by several workers (THIELE and SCHÜMANN 1942, SCHNETZ and FLUCH 1942) that the vasodilator imidazol "Priscol" (hydrochloride of 2-benzyl-4.5-imidazolin), which is related to histamine, stimulates acid secretion in animals and man. In three anaesthetized and vagotomized cats this drug was injected intravenously at a constant rate of 0.5—1.0 mg per kg and minute during 30—60 minutes. The rate of acid gastric secretion was high. The three specimens of native gastric juice contained 30, 45 and 65 γ histamine per litre. Adequately performed controls showed that "priscol", which might possibly enter the gastric juice from the blood, does not interfere with the histamine assay on the gut.

D. Rate of Secretion and Histamine Content of Juice.

In four cats both vagi were stimulated electrically during 30 minutes, and the juice corresponding to each period of stimulation tested directly on the gut for its histamine content. In a fifth cat secretion was induced by "priscol". The results are given in Table 6. The figures show that the histamine content of the juice remains rather constant independent of the secretion rate.

In seven cats gastric secretion was induced during 2 to 10 hours by various prolonged stimuli. At the beginning and end of these long periods the native gastric juice was tested for its

Table 6.

The histamine contents of gastric juice secreted at different rates.

Cat nr	Stimulus	Rate of gastric secretion, ml/30 minutes	Hi content γ per litre
1	Vagus stimulation	1.0	40
		4.8	45
2	»	1.3	40
		2.2	35
		2.8	40
3	»	2.4	35
		3.4	30
		5.2	35
4	»	5.5	30
		6.0	35
		9.0	35
		10.6	30
5	Injection of priscol	2.4	40
		7.7	50

Table 7.

The histamine content of gastric juice at the beginning and end of prolonged secretion periods.

Cat nr	Stimulus	Duration of stimulation hours	Gastric juice ml	Histamine content, γ per litre	
				Beginning	End
1	Vagus stimulation	6	25	40	40
2	»	10	50	35	35
3	»	2	8	40	35
4	»	4	63	35	35
5	Slow inj. of Hi	3	80	20	25
6	»	2	45	35	25
7	Slow inj. of priscol	4	50	40	35

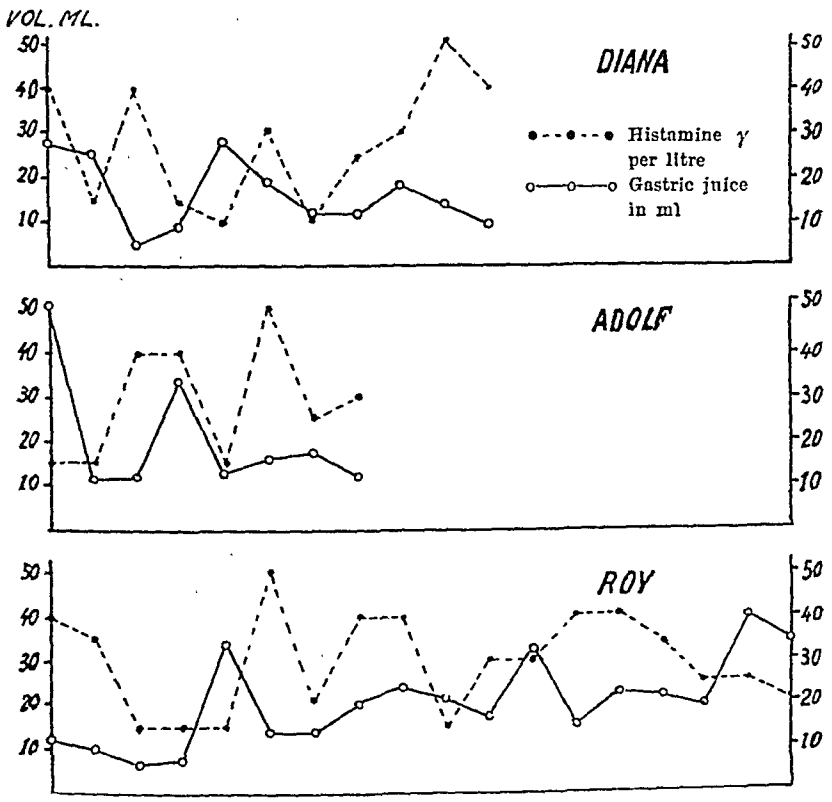


Fig. 7.

histamine content. The results are given in table 7. From this table it is seen that the histamine content of the juice is approximately the same at the beginning and the end of even very long periods of secretion.

In three dogs with gastric pouches gastric juice was collected during the gastric phase of secretion. From each dog a sample was collected daily on some day arbitraryly chosen during periods ranging up to five months. Fig. 6 shows that no definite relation exists between the rate of secretion and histamine content of the juice. The lack of correlation is also obvious from figures 1 and 2.

E. Results with Slow Injection of Histamine.

In these experiments on anaesthetized cats histamine was injected during subsequent definite periods intravenously at an increasing rate for each period. In previous experiments by EMMELIN et al. on dogs it was shown that the lowest rate of injection which causes a just detectable increase in the histamine content of plasma ranges between 2 and 5 γ per kg and minute. These authors also observed that the lowest rate of injection which stimulates parietal secretion amounts to about 0.5 γ per kg and minute. In the actual experiments referred to in table 8 the lowest rates of injection employed are well above these threshold.

Table 8.

The histamine content of gastric juice obtained on intravenous injection of histamine at increasing rate.

Cat nr	Rate of histamine injection, γ/kg and minute	Duration of injection minutes	Vol. of gastric juice in ml	Histamine content γ per litre
1	1	38	13.5	16
	20	40	10.5	30
	40	42	4.9	45
	100	18	1.8	95
2	1.2	25	7	60
	12	5	2	60
3	1	37	2	30
	22	38	3	45
4	1.6	35	5	55
	32	20	5	105
	65	25	0.4	160
5	1.5	10	3	20
	15	10	3.5	20
	60	15	2	55
6	2	10	5	25
	40	20	10	45
	80	17	6	75
	120	5	1	180
7	3	115	45	35
	40	12	25	45

The experiments presented in table 10 suggest that histamine, if present in a diffusible state in the surroundings of the parietal cells, enters these cells and appears in the gastric juice. Finally it is shown that histamine, which by these means finds its way to the gastric juice, appears there in about the same order of concentration as in juice obtained by the various other previously described modes of stimulation.

F. Different Stimuli with the same Animal.

In a special series gastric secretion was evoked by different stimuli applied to one and the same animal. The native juice corresponding to each type of stimulus was tested for its histamine content. Table 9 gives the data obtained from six cats under chloralose.

Table 9.

The histamine content of gastric juice obtained on different modes of stimulation.

Cat nr	Stimulus	Histamine content, γ per litre
1	Spontan. secretion	45
	Vagus stimulation	45
	Injection of priscol	45
2	Spontan. secretion	40
	Injection of "gastrin"	35
3	Injection of "gastrin"	45
	Vagus stimulation	40
4	Vagus stimulation	25
	Injection of priscol	30
5	Spontan. secretion	25
	Acetylcholine intraventr.	25
6	Spontan. secretion	40
	Acetylcholine intraventr.	40

Discussion.

Parietal secretion, no matter by what mode of stimulation the excitation process is induced, involves the liberation of histamine, as judged by the occurrence of histamine in the gastric juice. Considerable amounts of histamine are found in the gastric juice during the cephalic as well as during the gastric phases of secretion, and also in juice obtained with secretogogue drugs. Histamine occurs in the juice in a physiologically active state.

It thus seems reasonable to assume that the liberation of histamine in the fundic mucosa represents an integral link in the mechanism devised to excite the parietal cells.

The fundic mucosa in dogs and cats contains extraordinarily large amounts of histamine. There is no reason to believe that the histamine of the gastric juice originates from other sources. In many experiments the histamine content of gastric juice exceeds by far that of blood plasma, for which figures have been evaluated in dogs and cats by several workers (CODE 1937, MINARD 1941, EMMELIN, KAHLSON and WICKSELL 1941), and it is unlikely that the parietal cells concentrate the histamine present in their surroundings. The histamine concentration in the mucosa is high enough to ensure a relatively high histamine content of the gastric juice even during very long periods of secretion. — As pointed out by MACINTOSH (1938), the absence of histaminase in the gastric mucosa (BEST and MCHENRY, 1930) is favourable to the functions of histamine there, since no known enzyme interferes with the diffusion of histamine into the parietal cells and the acid juice, once it is liberated in their surroundings or within them.

From the experiments where acid secretion was stimulated by slow intravenous injections of histamine, it is obvious that histamine, if present in the surroundings of the parietal cells in a diffusable state, enters these cells and emerges in the acid juice. The histamine concentration in the juice is higher the higher the rate of intravenous histamine injection, i. e. the higher the concentration in the surroundings of the secreting cells. It seems reasonable to assume that on stimulating with histamine injections the histamine which enters and passes through the glands acts as a direct excitant. The question then arises whether the histamine concentration of juice from the cephalic and gastric phases is sufficient to excite the glands. Our experiments indicate an affirmative answer, since the histamine concentration of juice from the cephalic and gastric phases is of the same order as in juice obtained with injection of histamine.

With the exception of juice obtained with histamine injections where the histamine concentration of the plasma increases with higher rates of injection, there is no correlation between histamine concentration of the juice and rate of secretion. In the same animal, with different modes of stimulation, the histamine concentration of the juice remains rather constant, independent of the secre-

tion rate. This observation does not oppose the idea that histamine is the final link in exciting the parietal cells, since with an increasing rate of secretion a larger amount of cells might be activated, the individual gland yielding an acid juice with rather constant histamine concentration.

In some specimens of gastric juice the histamine equivalents were exceptionally low. This might be due to the fact that under some conditions which we cannot yet define the juice contains one or more agents which interfere with the assay. This agent relaxes smooth muscle and opposes the effect of histamine. It is not inactivated by the chemical treatment involved in the Barsoum-Gaddum-method. We are engaged on a closer study of this substance.

Histamine-free preparations of "gastrin" injected intravenously cause a liberation of histamine in the fundic mucosa, as judged by the occurrence of histamine in the gastric juice. In previous work from this laboratory (UVNÄS 1942) it was shown that the pyloric hormone "gastrin" is in some way or other also engaged in the cephalic phase of gastric secretion. As to the gastric phase, conclusive evidence of the intervention of "gastrin" is now available (GREGORY and IVY 1941). In view of these facts it is tempting to assume that, during the cephalic and gastric phases, parietal secretion is excited by a two-stage humoral mechanism, the first stage involving the liberation of "gastrin", and histamine representing the second, final link. This conception assumes that a chemical agent is capable of liberating histamine. The observation that histamine is liberated by the secretogogue drug "priscol" is not unfavourable to this view.

Summary.

1. In cats and dogs acid gastric juice contains histamine in a physiologically active state.

2. The histamine content of gastric juice is independent of the mode of stimulus employed in exciting the parietal cells, thus being of the same order in juice from the cephalic and gastric phases and in juice obtained by injection of secretogogue drugs.

3. The histamine concentration of the juice secreted during the cephalic and gastric phases is sufficient to stimulate the parietal cells.

4. Histamine-free preparation of "gastrin" cause a liberation of histamine from the fundic mucosa as judged by the occurrence of histamine in the acid juice.

5. It is suggested that in the humoral mechanism instrumental in exciting the parietal cells in the cephalic as well as in the gastric phase of acid secretion histamine represents the second, final link.

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The Amino Acid Composition of Whole Fish Protein Present in Some Species of Swedish Fish.

By

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In connection with the researches made in this laboratory for the purpose of throwing some light on the Chastek paralysis (ÅGREN 1944, LIECK and ÅGREN 1944) it was decided to determine the amino acid composition of whole fish protein present in some Swedish fishes which either had not yet been analyzed at all or which had been investigated by less modern and specific methods than the one which was intended to be used in the present investigation. Since ROSE (1938) had shown that animals deprived of valine exhibited unusual symptoms including sensitiveness to touch and absence of muscular coordination, symptoms, which also are exhibited by foxes suffering from Chastek paralysis, special attention was given to the determination of this amino acid. As is known the disease will follow the incorporation of raw fish in the ration of foxes and as this frequently is done in Sweden, the endemic occurrence of Chastek paralysis in this country (CARLSTRÖM and RUBARTH 1943) is no surprising phenomenon.

The material was prepared for analysis by boiling the fresh fish for 20 minutes and by freeing it from bones. The fishes were reduced to a pulp by means of a meat chopper. The pulp was extracted three times with two times its volume of alcohol and then with ether until it was freed from the major part of fat.

The product was air-dried and powdered by treatment in a rolling-mill for 24 hours. After this procedure it was extracted in a Soxhlet apparatus for 24 hours and air-dried.

Results and Discussion.

The nitrogen distribution was determined by the electrodialysing technique as modified by THEORELL (1943). The values given in Table 1 are typical of the different fractions. For a comparative study, the values obtained on the muscle proteins present in the same species of fish are also indicated.

Table 1.

The Nitrogen Distribution of Fish Protein.

All values in per cent of total nitrogen.
W. P. = Whole fish protein. M. P. = muscle protein.

Fraction	The sprat		The roach		The cod	
	W. P.	M. P.	W. P.	M. P.	W. P.	M. P.
Humin-N	1.8	1.6	1.9	1.1	1.8	1.5
Amide-N	9.8	11.0	10.5	10.4	7.8	8.0
Anodic-N	14.8	12.6	14.0	11.4	14.0	12.4
Neutral-N	41.5	45.5	41.5	48.0	44.6	45.0
Cathodic-N	28.3	25.0	27.6	22.4	28.0	27.8

The results are in good agreement with those obtained by ROSEDALE (1929) in other species of fish as well as with those obtained by ÅGREN (1944) on the muscle proteins from the same species of fishes. Table 2 shows the result of the amino acid determinations on whole fish protein as well as those of a previous analysis of the muscle proteins present in the same species of fish. The indicated valine values of the muscle proteins are the re-investigated values (v. experimental part).

Table 2 demonstrates that from a nutritional point of view there is but an insignificant difference between the data obtained in the present investigation and the values obtained on the muscle proteins that were present in the same series of fish. All the amino acids which ROSE (1938) considered to be indispensable owing to their property of stimulating growth seem to be present. (Isoleucin was not determined). Especially may be mentioned that considerable amounts of both glycine, arginine and methionine were determined. These three amino acids are taking part in the synthesis of creatin, which is of essential im-

Table 2.

The Amino Acid Composition of Fish Protein.

The values are expressed as percentage of the moisture and ash free protein. W. P. = Whole fish protein. M. P. = Muscle protein.

Amino acid	Sprat		Roach		Cod	
	W. P.	M. P.	W. P.	M. P.	W. P.	M. P.
Arginine	8.10	7.20	7.40	7.30	9.10	9.0
Histidine	1.45	1.70	1.37	1.45	1.50	1.60
Lysine	7.26	6.0	5.0	4.10	4.62	4.80
Hydroxylysine	1.64	1.40	1.32	1.30	0.68	1.15
Tyrosine	3.81	3.60	3.30	4.60	4.0	4.75
Tryptophane	0.73	0.85	0.65	0.68	0.69	0.67
Proline	7.80	5.0	8.30	7.10	6.50	5.10
Hydroxyproline	0	0	0	0	0	0
Threonine	0.52	0.58	0.45	0.58	0.41	0.57
Serine	3.45	2.45	3.15	4.45	3.30	3.50
Glycine	0.70	1.81	0.33	2.70	0.94	1.0
Phenylalanine	12.0	14.0	12.3	14.8	13.3	14.4
Cystein + cystin	1.50	1.60	1.60	1.60	2.0	1.80
Methionin	2.40	2.30	2.30	2.20	2.40	2.20
Leucine	11.6	15.5	11.0	18.0	12.6	16.8
Valine	6.9	7.4	7.2	7.1	7.1	7.4
Hydroxyglutamic acid	0	0	0	0	0	0
Aspartic acid / Glutamic acid	24.8	24.4	21.7	20.4	22.1	22.3
Ammonia	1.80	1.95	1.60	1.80	1.70	1.50
	96.5	97.7	89.0	102.2	92.9	98.5

portance in muscle metabolism. A decrease of creatin values of the muscle is considered to be connected with muscle degeneration, which is also one of the symptoms in the Chastek paralysis.

The question whether the different amino acids in whole fish protein are present in sufficient amounts to satisfy the synthesis of body proteins in animals cannot be solved by merely analyzing the amino acids of fish. But on the basis of a comparison between the amino acids present in whole fish protein with the amino acid composition of the rabbit muscle (SHARP, 1939) it will be possible to draw some conclusion. SHARP's values are given in Table 3.

Table 3.

The Amino Acid Composition of Rabbit Muscle.

The values are expressed in percentage of moisture and ash free protein.

arginine	7.0	phenylalanine	2.0
histidine	1.7	cystin	0.77
lysine	9.9	methionine	3.40
tyrosine	3.4	leucine	7.1
tryptophane	0.89	valine	2.2
proline	0.4	aspartic acid / glutamic acid	22.1
threonine	1.2		

On comparing Table 2 with Table 3 it will become obvious that the amino acids in fish protein can furnish sufficient material for the synthesis of animal muscle protein, at least as far as rabbits are concerned. Attention should be drawn to the fact that the amounts of valine present in the fish protein are of the same order of size as those present in rabbit muscle protein. ROSE (1938) reported that animal deprived of valine exhibited unusual symptoms including sensitiveness to touch and lack of muscular coordination. These symptoms are also manifested by foxes suffering from Chastek paralysis, but it is not likely that the symptoms are due to lack of valine in the fish proteins included in the ration of foxes.

Experimental.

Moisture, ash, total nitrogen, nitrogen distribution, amide nitrogen and humin nitrogen were determined by the methods given in a previous paper (ÅGREN 1944). Of the amino acid methods only the valine method was more extensively studied in the present investigation.

Determination of valine. As was pointed out in the discussion, special attention was given to the determination of the valine values in the present investigation. In view of this and also of the fact that no data as to the application of the colorimetric valine method of WRETLIND (1943) to protein hydrolysates are as yet available, the method was further investigated. As some of the chemicals requested for the determinations were short in supply owing to the war conditions, it was necessary to determine as small amounts of valine as possible. Using 3 ml of a total of 10 ml of steam distillate it was found that the empirical correction factor of WRETLIND was not constant but varied in the following way, if the added amounts of valine should be regained:

valine in γ	250	100	50	25
extinction value	0.30	0.17	0.11	0.06
correction factor	250	180	140	140

Each extinction value is the mean of 5 determinations. Using 1 cm cups in the Zeiss Photometer, 250—50 γ of valine were determined with an accuracy of $\pm$ 10 %. From the extinction values a curve was constructed from which the correction factor of valine values between 250—50 γ could easily be read off. For determination of 25 γ and less, the colorimetric reaction was carried out on 6 ml of a total of 10 ml of steam distillate, 4 ml 10.5 N sodiumhydroxide and 1 ml 20 % by volume salicylic-aldehyde. The extinction values and correction factors obtained when using 3 cm cups in the photometer, are given below.

valine in γ	50	25	15
extinction value	0.26	0.13	0.08
correction factor	115	115	114

In this way it was possible to determine as small amounts as 15 γ of valine with an accuracy of $\pm$ 10 %. The next step in the investigation was the testing of the method on hydrolysates of proteins which had previously been analyzed with the Fischer-estrification method. According to SCHMIDT (1938) casein contains 7.9 % of valine whereas gelatin does not contain any valine at all. According to SHARPENEK et al. (1934) and SHARP (1939) mammal muscle protein contains considerable amounts of valine. Therefore the valine amounts of casein, gelatin and cattle muscle protein were investigated by WRETLIND's method.

The proteins were hydrolyzed and leucine precipitated from the hydrolysates by the same procedure as described by ÅGREN in 1944. The combined filtrates and washings from the leucine precipitation were used for the analysis. In preliminary experiments it was found that if valine was added to the hydrolysates it was quantitatively recovered. The determinations of the valine content of caseine, gelatin and muscle protein were carried out directly on the hydrolysates as well as on hydrolysates electrodialyzed with the apparatus described by THEORELL (1943). Samples of 150 mg of hydrolyzed protein were electrodialyzed for 12 hours. Ammonia was freed from the cathode fraction by distillation and the remaining solution re-electrodialyzed. The neutral fraction from the re-electrodialysis was added to the first neutral fraction. The combined neutral fractions from 300 mg of electrodialyzed protein were concentrated to 5 ml and used for the analysis. The determinations on hydrolysates and electrodialyzed hydroysates yielded the following typical values:

	Casein	Gelatin	Muscle protein
Hydrolysates	7.0	5.5	6.0
Electrodialyzed hydrolysates . . .	7.1	5.4	5.8

All values are given in per cent of ash and moisture free protein.

The values demonstrate in a satisfactory manner that there is no loss of valine from the middle compartment of the apparatus during the electrodialysis. The casein values are in good agreement with those which a previous analysis (SCHMIDT, 1839) yielded. The presence of valine in gelatin was rather surprising and therefore the casein and gelatin values were controlled by running 1 ml of the concentrated neutral fractions of the electrodialyzed hydrolysates through the TISELIUS adsorption analysis apparatus. The procedure used in the analysis permits, after adsorption of a mixture of neutral amino acids, the separation of neutral amino acids of higher molecular weight as for instance valine and leucine from acids of low molecular weight by the displacement development of the adsorption column (TISELIUS, 1944)[1]. In this special case the preliminary precipitation of leucine from the hydrolysates facilitated the analysis. The diagrams

[1] The adsorption analyses were carried out by Prof. TISELIUS in the Department of Physical Chemistry, University of Uppsala.

drawn from the adsorption analysis of electrodialyzed casein also demonstrated the presence of only one substance in the fraction which could be suspected to contain amino acids of high molecular weight. A colorimetric analysis of this fraction gave a value corresponding to 5.7 % of valine in casein, the fraction containing amino acids of low molecular weight such as glycin, alanine, prolin, oxiprolin and serine did not give any color reaction. In this case the two methods yielded similar results.

The adsorption analysis of electrodialyzed gelatin gave a diagram showing one rather low step in the fraction of amino acids of high molecular weight and in agreement with this the colorometric method gave a value corresponding to 0.25 % of valine in gelatin. A colorimetric analysis was also carried out on the fraction containing the amino acids of low molecular weight and the value obtained in this fraction corresponded to 1.9 % of valine in gelatin. The combined values of the two fractions thus corresponded to 2.2 % of valine in gelatin. The reason of this discrepancy between the adsorption analysis of casein and that of gelatin may be due to the fact that in gelatin more than half of the molecule is built up by 3 low molecular amino acids namely glycin, prolin and oxiprolin. During the displacement development the presence of such a high concentration of low molecular amino acids may cover a part of the valine fraction in the diagram and a complete separation of the valine-leucine fraction from the fraction containing the neutral amino acids of low molecular weight is therefore not possible.[1]

After these preliminary investigations the valine content of fish proteins were analyzed. The determinations were carried out directly on hydrolysates as well as on electrodialyzed hydrolysates. Leucine was precipitated from the hydrolysates as previously described. Typical results of the analyses are given below.

	Cod		Sprat		Roach	
	M. P.	W. P.	M. P.	W. P.	M. P.	W. P.
Hydrolysates	7.4	7.2	7.0	6.8	7.4	6.5
Electrodialyzed hydrolysates	7.2	7.3	6.8	6.9	7.0	6.2

All values are given in per cent of ash and moisture free protein.
M. P. = muscle protein. W. P. = Whole fish protein.

In agreement with the results obtained when investigating casein and gelatin, the values demonstrate that no loss of valine ocurred during the electrodialysis. The valine values of the fish muscle protein were not in agreement with previously obtained results (ÅGREN, 1944). This is due to the fact that old ninhydrin samples, obviously of low quality had to be used in the previous analysis, as no other samples were awailable at that stage of the investigation.

[1] After this manuscript had been finished, it came to the author's knowledge that the presence of valine in gelatin had recently also been demonstrated by KUIKEN et al. (1943) by means of a microbiological method.

Summary.

The amino acid composition of the whole fish protein obtained from some Swedish fishes which are commonly added to the ration of foxes was investigated. By incorporation of alanine values obtained by other authors, more than 100 per cent of the amino acid content was recognized. The nutritional value of the whole fish protein must be classified as rather high, as — with exception of isoleucine which was not analyzed — it was found that all indispensable amino acids were present in amounts which correspond to those reported to be present in human and rabbit muscle protein. The application of the colorimetric valine method of WRETLIND on protein hydrolysates was thoroughly investigated.

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Close Arterial Injection of Adenosine Triphosphate and Inorganic Triphosphate into Frog Muscle

By

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In the preceding communication (BUCHTHAL et al. 1944) the striking effect of micro-application of adenosine triphosphate (ATP) and related compounds has been described, ATP releasing mechanical responses, changes in birefringence and action potentials. Apart from a paper by ABDON (1942), who applied ATP intra-arterially to the gastrocnemius of the frog, no other investigation concerning the effect of this substance on striated muscle seems to exist. In contrast to the observations by BUCHTHAL et al., ABDON could not observe any stimulating action of ATP and found only inhibiting effects on the release of contraction by acetylcholine. We therefore thought it of interest to investigate anew the effect of intra-arterial application of ATP to frog muscle.

Method.

The method used for intra-arterial injection was about the same as that used by BROWN (1937) for the investigation of the effect of acetylcholine on frog muscle. All branches of the sciatic artery except those supplying the gastrocnemius musle were ligated. As the frogs available (Rana esculenta and Rana temporaria) were only $^1/_3$ the size of those used by BROWN, a fine glass cannula with an opening of 110 μ was inserted in the sciatic artery instead of a hypodermic cannula, the gastrocnemius muscle being fixed in a horizontal position. The weight of the muscles used was 300 to 400 mg.

[1] Working on a fellowship from the *Rockefeller Foundation*.

ATP and inorganic triphosphate were applied in an iso-osmotic condition and with a pH of 7.3. The preparation of the substances which were kindly provided by Dr. A. DEUTSCH (Research laboratory, A. B. Leo, Hälsingborg), is described in the preceding paper. The staple solution of ATP contained 4.35 mg/ml and that of inorganic sodium tri-poly-phosphate 2.9 mg/ml. All arterial injections were made in a volume of 0.03 ml Ringer. The composition of the Ringer's solution was as follows: 6.5 g NaCl, 0.2 g KCl. 0.14 g anhydrous $CaCl_2$ and 0.2 g glucose, distilled water to 1 litre. By a suitable amount of $NaHCO_3$ and by passing a gas mixture of 1 per cent CO_2 and 99 per cent O_2 through the solution its pH was adjusted to 7.3. The experiments were performed at 18—19° C. The mechanical responses were either registered isometrically or isotonically aud in some experiments action potentials were simultaneously recorded by leading off with Ag-AgCl electrodes to an A.—C. amplifier and electrostatic oscillograph.

Results.

Intra-arterial injection of *ATP* into the sciatic artery supplying the gastrocnemius of the frog sets up a tetanus-like contraction, the threshold amount being approximately 20 μg in 0.03 ml Ringer (0.04×10^{-6} mol). The tension produced by 40 μg ATP (0.08×10^{-6} mol) corresponds on an average to that released by 1 μg acetylcholine. After injection of ATP tension rises quickly and is accompanied by an outburst of asynchronous action potentials. The contraction may be maintained over a considerable time, and the persisting tension, too, is accompanied by electrical activity (Fig. 1 B). Tension increases with increasing concentration of ATP.

When acetylcholine is applied after previous injection of ATP, the sensitivity of the muscle to acetylcholine is essentially increased (Fig. 1 C). The effect of acetylcholine after previous application of ATP is 4-10 times that before the use of it (Figs. 1 and 2).

Inorganic tripolyphosphate likewise initiates tetanic contraction. The mechanical response indicates here a materially more asynchronous activity of the different fibres than is the case after application of ATP or acetylcholine. The abrupt rise in tension following injection of triphosphate is superposed by a diffuse fibrillary activity (Fig. 3 B). The threshold amount is somewhat higher than for ATP, 50-60 μg triphosphate (0.1—0.13×10^{-6} mol) releasing a mechanical response with the same tension as that initiated by 1 μg acetylcholine.

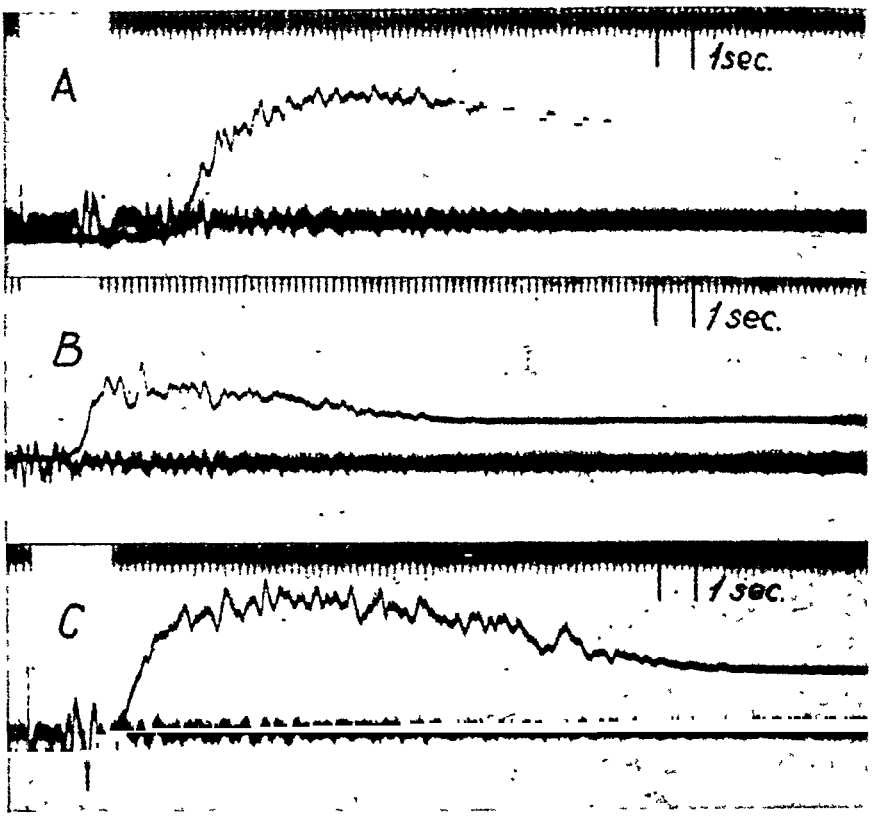

Figure 1. Action potentials (upper curves) and mechanical tension (lower curves) of the frog gastrocnemius after close arterial injection of:

A. 6 μg acetylcholine in 0.03 ml Ringer.
B. 217 μg sodium adenosine triphosphate (0.44×10^{-3} mol) in 0.03 ml Ringer
C. 0.6 μg acetylcholine in 0.03 ml Ringer after previous application of adenosine triphosphate (B).

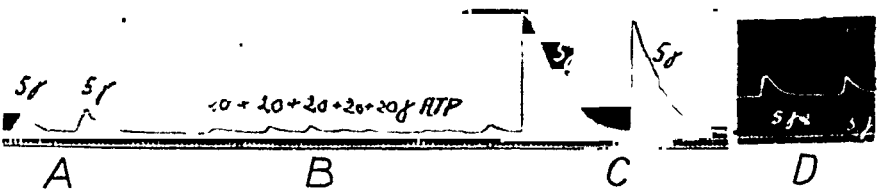

Figure 2. Mechanical tension of the frog gastrocnemius muscle after arterial injection of:

A. 5 μg acetylcholine in 0.03 ml Ringer.
B. 20 μg sodium adenosine triphosphate (0.04×10^{-3} mol, applied 5 times in 0.03 ml Ringer).
C. 5 μg acetylcholine in 0.03 ml Ringer after preceding injection of adenosine triphosphate (B).
D. 5 μg acetylcholine 4.5 min after C.
Time marks: 1 sec.

In a series of experiments made at another time of the year the sensitivity of the muscle to acetylcholine and triphosphate was essentially higher (c. 20 times), the threshold for acetylcholine being 0.025 μg.

Application of acetylcholine after previous treatment of the preparation with triphosphate — as after ATP — releases a considerably higher tension than before application of the phos-

Figure 3. Intra-arterial injection of:

A. 0.6 μg acetylcholine in 0.03 ml Ringer.
B. 58 μg (0.13×10^{-3} mol) sodium tripolyphosphate in 0.03 ml Ringer.
C. 0.6 μg acetylcholine in 0.03 ml Ringer after preceding application of sodium tripolyphosphate (B).
Time marks: 1 sec.

phate (Fig. 3 C). Furthermore, the responses produced by triphosphate are in their turn enhanced by previous application of ATP.

It can be seen from these experiments that close arterial injection of ATP and inorganic triphosphate in relatively low concentrations releases a tetanus-like contraction in the gastrocnemius muscle of the frog, and they furnish further evidence that ATP is an important agent in the initiation of contraction. When the reaction to acetylcholine is used as a basis for comparison, frog muscle is about 3—4 times more sensitive to ATP than mammalian muscle (Buchthal and Kahlson 1944). The negative results obtained by Abdon (1942) with injection of adenosine triphosphate by means of a similar technique to that used here are difficult to explain. The doses of ATP apparently correspond to those used in our experiments. A factor involved may, however, be the acetylcholine contracture produced before ATP is applied by adding 200—300 mg acetylcholine to the surrounding bath.

Summary.

1. In the gastrocnemius of the frog close arterial injection of small amounts of adenosine triphosphate and inorganic triphosphate evoke tetanus-like contractions. The mechanical response produced by 40 μg ATP (0.08×10^{-6} mol) or 50—60 μg triphosphate (0.10—0.13×10^{-6} mol) corresponds to that released by 1 μg acetylcholine.

2. The sensitivity of the preparation to acetylcholine and inorganic triphosphate is greatly increased by previous application of adenosine triphosphate.

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From the Department of Physiology, University of Lund.

The Action of Adenosine Triphosphate and Related Compounds on Mammalian Skeletal Muscle.[1]

By

FRITZ BUCHTHAL[2] and GEORG KAHLSON.

Received 8 July 1944.

In experiments on isolated muscle fibres of the frog it has been shown in a preceding paper (BUCHTHAL et al. 1944) that adenosine triphosphate (ATP) and some related compounds exert two different actions. ATP releases contraction in extremely small amounts and causes long-lasting changes in birefringence which are interpreted as an expression of restitutional processes in the contractile protein molecule. In accordance with NEEDHAM et al.'s suggestion founded on model experiments with myosin, by themselves (1941, 1942) and by ENGELHARDT and LJUBIMOVA (1939), observations on *living* muscle fibres of the frog support the idea that ATP is the physiological agent engaged in the release of contraction.

It lay close to hand to investigate how far this theory applies to mammalian muscle in order to obtain a broader experimental background and — if possible — to establish a generalisation of the interaction between ATP and living muscle. The action of ATP and related phosphates on mammalian muscle has — as far as we know — not yet been investigated. As it is well known that the mode of application of active substances may decisively influence the pattern of response in skeletal muscle, we apply the most physiological way of providing contact between the

[1] A preliminary account of this work was sent to *Nature* on June 6th, 1944.
[2] Working on a fellowship of the *Rockefeller Foundation*.

substances in question and muscle, and use the method of close arterial injection as devised by BROWN et al. 1936.

Method.

The experiments were performed on the tibialis anterior muscle of decerebrated cats as described by BROWN (1938). 21 animals generally weighing 2.8—3.5 kg with a weight for the tibial muscle of 6—7 g were used. Injections are made in the distal part of the anterior tibial artery, the proximal part being temporarily closed by traction on a ligature, when the substances are applied. The most suitable injected volume was found to be 0.5 ml and an equal amount of Thyrode solution is injected immediately after application of the active substance.

Tension is recorded isometrically with the BROWN-SCHUSTER flat-spring myograph. The excursions of the spring are transmitted by a torsion-band with mirror attached to the steel spring of the myograph. Simultaneously with tension *action potentials* are recorded by leading off with three concentric needle electrodes to an A. C. amplifier and electrostatic oscillograph. The three electrodes are introduced to different parts of the muscle and connected in parallel to the same amplifier. When substances are applied by close arterial injection in a volume of 0.5 ml, they generally reach only certain portions of the muscle as controlled by injection of corresponding amounts of Indian ink at the end of each experiment. By using three electrodes the chance of leading off from active portions is of course greatly increased.

Care was taken that rectal temperature and temperature of the muscle were approximately 37° C. In a special serie of experiments muscle temperature was only 20° C.

Every substance was tested on the curarised and non-curarised animal. Curare was applied intravenously in the jugular vein or intra-arterially in the anterior tibial artery in such amounts that maximal electrical stimulation of the sciatic nerve and large doses of acetylcholine were ineffective.

All substances were applied iso-osmotically by substituting an equivalent amount of NaCl + water in the Thyrode solution with a staple solution of the substance in question. The pH of the injected solution was 7.3, and its temperature 37° C.

The following substances were applied as sodium salts, and their preparation is described in the paper by BUCHTHAL et al. (1944). They were kindly provided by Dr. A. DEUTSCH (Research Laboratory, A. B. Leo, Hälsingborg).

1. ATP, a 99 per cent preparation, staple solution 15 mg/ml (calculated as free acid).
2. Adenosine-diphosphate (ADP) staple solution 7.4 mg/ml.
3. ADP + orthophosphate » » 10.5 + 4.4 mg/ml
4. Adenylic acid » » 3.8 mg/ml
5. Adenylic acid + pyrophosphate » » 3.8 + 4.8 mg/ml

6. Inorganic tri-polyphosphate	staple solution		9.4 mg/ml
7. Pyrophosphate	»	»	10.9 mg/ml
8. Orthophosphate	»	»	4.4 mg/ml.

In the non-curarised animal the effect of the different substances was matched against the action of acetylcholine on muscle which was injected in amounts of 0.2—2 μg per g muscle.

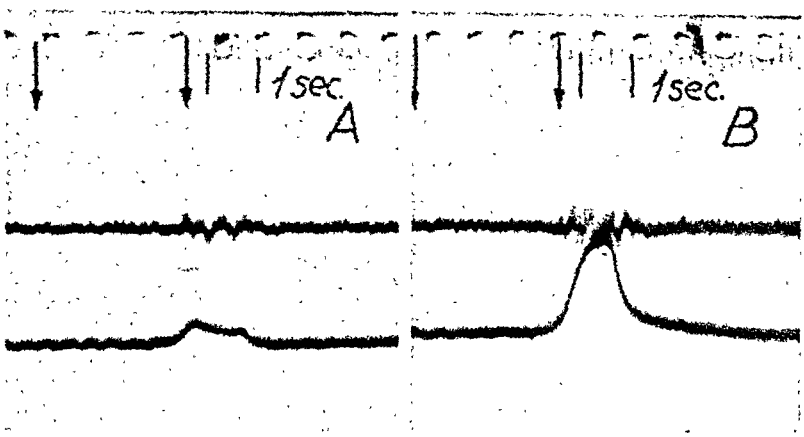

Fig. 1. Action potentials (upper curves) and mechanical tension (lower curves) of the anterior tibial muscle of the cat after close arterial injection of:

A. 0.1×10^{-6} mol/g muscle sodium adenosine triphosphate.
B. 0.2×10^{-6} mol/g muscle sodium adenosine triphosphate. Injection between arrows.

Results.

1. *Adenosine triphosphate (ATP).*

Close arterial injection of ATP in amounts of 0.05—0.53 mg/g muscle (1.46—14.6 $\times 10^{-6}$ mol/ml = 0.1—1.0 $\times 10^{-6}$ mol/g muscle) initiates a tetanus-like contraction. Tension develops abruptly after a latency period of approximately the same duration as that seen with acetylcholine. The smallest amount evoking contraction is c. 0.1 $\times 10^{-6}$ mol/g muscle (Fig. 1). The tetanic nature of the contraction is obvious from the accompanying action potentials and the electrical activity persists as long as tension is maintained (Fig. 2). The typical mechanical and electrical response to ATP can be reproduced 4—5 times, when injections are made at intervals of a few minutes. Then the sensitivity to ATP (and to acetylcholine) decreases successively but reaches nearly the previous level after a pause of c. 10 minutes.

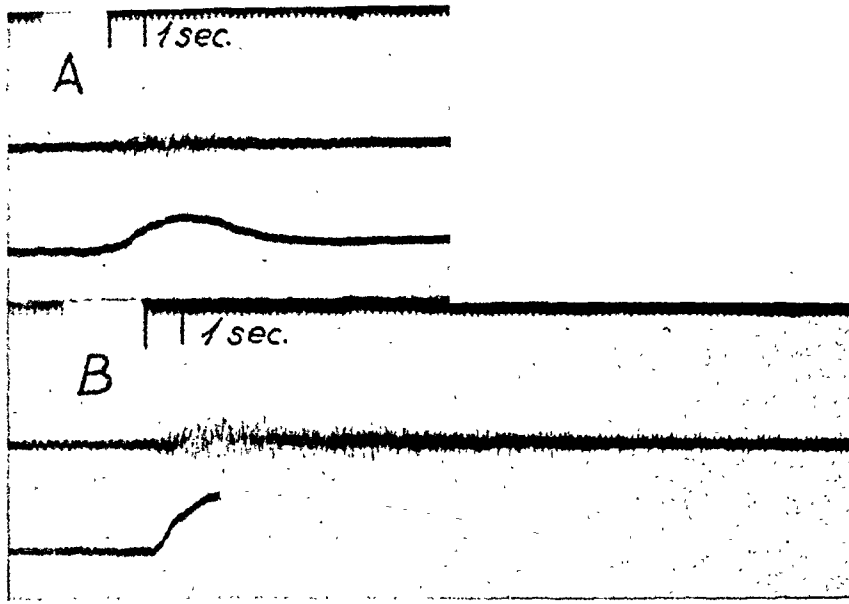

Fig. 2. Action potentials (upper curves) and mechanical tension (lower curves) after intra-arterial injection of:

A. 0.22×10^{-6} mol/g muscle sodium adenosine triphosphate.
B. 0.6×10^{-6} mol/g muscle sodium adenosine triphosphate.
Interruption of time marks denotes the duration of the injection.

With ATP there exists a definite relation between dose and effect, tension increasing steeply in height and duration with increasing dosage. The tension produced by c. 140 μg ATP corresponds to that released by 1 μg acetylcholine in the non-curarised muscle. The reaction of mammalian muscle to ATP is not influenced by curarisation.

With decreasing temperature of the muscle the mechanical responses elicited by ATP and acetylcholine are considerably prolonged.

ATP augments the response to acetylcholine.

There is a striking difference in the mechanical response of the muscle to acetylcholine before and after application of ATP. The short tetani elicited by small amounts of acetylcholine are enhanced in height and especially in duration, when the muscle has previously been treated with ATP, and the acetylcholine response resembles in duration that evoked by a single injection of ATP (Fig. 3).

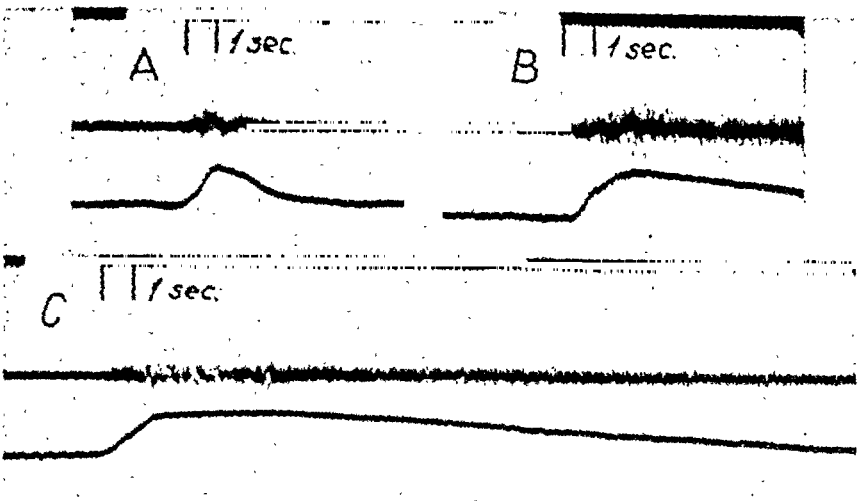

Fig. 3. Augmentation of the effect of acetylcholine after preceding application of sodium adenosine triphosphate.

A. 0.7 μg/g muscle acetylcholine.
B. 0.6 × 10^{-6} mol/g muscle sodium adenosine triphosphate.
C. 0.7 μg/g muscle acetylcholine after preceding injection of adenosine triphosphate (B).
Upper curves: action potentials.
Lower curves: mechanical tension.
Interruption of time marks denotes the duration of the close arterial injection.

2. *Adenosine diphosphate* (ADP) has essentially the same action on mammalian muscle as ATP. The substance is effective from amounts of 1.7 to 7.0 × 10^{-6} mol/ml = 0.12—0.5 × 10^{-6} mol/g muscle and evokes a short tetanus-like response. An equimolar mixture of ADP + orthophosphate (3) is more active than the same amount of ADP alone (7.0 + 7.0 × 10^{-6} mol/ml = 1.0 + 1.0 × 10^{-6} mol/g muscle). In this respect mammalian muscle differs from frog muscle, where addition of orthophosphate does not augment the action of ADP. In interpreting the potentiation, it is of interest to note that mammalian muscle is entirely insensitive to orthophosphate (see pg. 322).

4. Close arterial injection of *adenylic acid* in concentrations of 0.58—8.1 × 10^{-6} mol/ml = 0.04—0.58 × 10^{-6} mol/g muscle evokes brief tetanic contractions in curarised and non-curarised mammalian muscle. In frog muscle this substance neither initiates contraction nor alters birefringence. The duration of the response caused by adenylic acid is almost as brief as that elicited by acetylcholine (Fig. 4). When, however, *adenylic acid* is applied together with *pyrophosphate* (5), ((0.58 + 0.58) × 10^{-6} mol/ml =

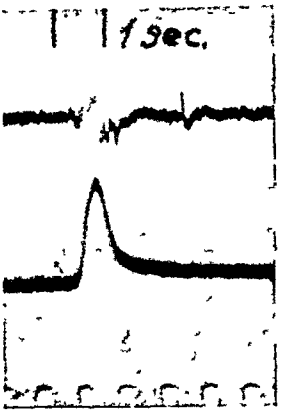

Fig. 4. Action potentials and mechanical tension after close arterial injection of 0.04×10^{-6} mol/g muscle adenylic acid.

$(0.04 + 0.04) \times 10^{-6}$ mol/g muscle) protracted tetanic contractions of the ATP type occur. As will be discussed in detail, the addition of pyrophosphate diminishes the response to all subsequent injections of active substances.

Apart from these organic phosphates the effect of the following inorganic phosphoric compounds on cat's muscle has been investigated:

6. *Inorganic sodium tripolyphosphate* is highly effective as a chemical stimulus. The substance evokes long-lasting, tetanic activity and was applied in amounts of $2—12 \times 10^{-6}$ mol/ml $= 0.15—0.9 \times 10^{-6}$ mol/g muscle (Fig. 5). As with ATP, the response increases with increasing con-

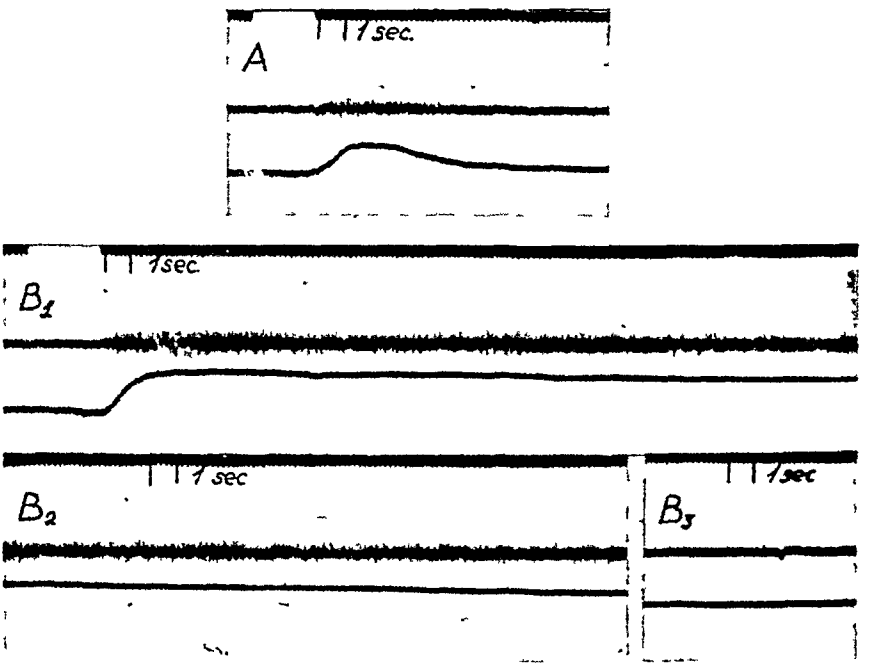

Fig. 5. Action potentials and mechanical tension after injection of:

1. 0.28×10^{-6} mol/g muscle sodium tripolyphosphate.
B_1. 0.56×10^{-6} mol/g muscle sodium tripolyphosphate.
B_1. continuation of B_1.
B_1. 25 sec after B_1.
Interruption of time marks denotes the duration of the close arterial injection.

centration, the concentration action curve being less steep than for ATP, and tension increases about proportionally with dosage.

7. *Sodium pyrophosphate* in amounts of $2.5—17 \times 10^{-6}$ mol/ml $= 0.18—1.25 \times 10^{-6}$ mol/g muscle releases a strong mechanical response of long duration which increases with increasing concentration as long as small quantities are applied. Since however the very first injection of pyrophosphate has a damaging effect on muscle, the second injection of an even larger dose very often releases smaller responses than the preceding amount.

8. *Sodium orthophosphate* in concentrations of 20×10^{-6} mol/ml $= 1.7 \times 10^{-6}$ mol/g muscle does not cause contraction in mammalian muscle.

Discussion.

The experiments described in this paper indicate that in mammalian muscle too, apart from its function as deliverer of energy for the restitution after contraction, adenosine triphosphate is engaged *in the primary release of contraction.* When the response to definite amounts of acetylcholine is used as a gauge of sensitivity in the non-curarised preparation, frog's muscle has been found to be 3—4 times more sensitive to ATP than mammalian muscle.

There is however an essential difference in the reaction of frog's muscle and the muscle of the cat. While in the former only the highly-energetic phosphate bonds act as chemical stimuli, in mammalian muscle *adenylic acid* too is effective in the initiation of contraction.

As regards the interesting interrelation between the action of ATP and acetylcholine, the experiments suggest that a stimulus either electrical or chemical is potentiated, when ATP is present in an accessible form in the surroundings of the contractile substance. In frog's muscle ATP releases a short tetanus with following persistant twitch-like activity. The tendency to a repetitive action apparently inheres for a considerable time, when the muscle has once been treated with ATP, and may be released by any subsequent stimulus.

Summary.

1. Close arterial injection of relatively small amounts of adenosine triphosphate (ATP) in the tibialis anterior muscle of the cat releases a tetanus-like contraction. The effective amounts range between 0.1—1.0 $\times 10^{-6}$ mol/g muscle, and the response increases steeply with the injected quantity.

2. The response to intra-arterially applied acetylcholine is greatly augmented by previous application of ATP.

3. Adenosine diphosphate (ADP) and ADP + orthophosphate likewise act as chemical stimuli, the effect of the latter being larger than with equal amounts of ADP alone.

4. Adenylic acid elicits a short tetanic contraction in amounts of 0.04—0.58 $\times 10^{-6}$ mol/g muscle, while it has no effect on frog's muscle. Adenylic acid + pyrophosphate causes a protracted tetanic contraction.

5. Sodium tripolyphosphate and pyrophosphate are highly active as chemical stimuli, while orthophosphate in amounts of 20 $\times 10^{-6}$ mol/ml (= 1.7 $\times 10^{-6}$ mol/g muscle) is ineffective. Pyrophosphate, however, reduces the excitability for all subsequent stimuli.

6. The active substances (apart from acetylcholine) evoke contraction also after complete curarisation of the muscle.

This work has been supported by a grant from the *Nobel Foundation.*

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From the Department of Physiology, University of Lund.

The Motor Effect of Adenosine Triphosphate and Allied Phosphorus Compounds on Smooth Mammalian Muscle.[1]

By

FRITZ BUCHTHAL[2] and GEORG KAHLSON.

Received 8 July 1944.

In view of the recent findings on striated muscle (BUCHTHAL et al. 1944) which supply evidence for the assumption that adenosine triphosphate (ATP) is an important agent engaged in the physiological release of contraction, it is of interest to extend the investigation concerning the interaction between myosin, skeletal muscle and phosphorus compounds to smooth muscle. Furthermore, the examination of smooth muscle with regard to its reaction to adenosine triphosphate and related compounds readily suggests itself, as the basic metabolic processes are supposed to be qualitatively identical in both substrates.

In the literature a number of partly conflicting observations exist on the effect of ATP and other adenylic compounds. With the intestines of the rabbit and the cat some authors find an inhibition of motor activity and a decrease of "tone" after application of ATP (DRURY and SZENT-GYÖRGYI 1929, ZIPF 1931, EULER, EULER and SCHLENK 1940, ABDON 1942 a. o.); others describe a brief decrease with a following increase in "tone" (GILLESPIE 1934). A systematic investigation concerning the specificity of the effect of ATP and other phosphate compounds does not exist.

[1] A preliminary account was sent to *Nature* on June 6th, 1944.
[2] *Working on a fellowship from the Rockefeller Foundation.*

The aim of the present investigation is to study by adequate technique the effect of the substances, the action of which was previously examined on mammalian and amphibian skeletal muscle.

Method.

Three specimens of smooth muscle were employed: the muscular coat of the cat's stomach, the cat's bladder, and the small intestine of the guinea pig. In the cat the substances were applied by the technique of close arterial injection, while in the guinea-pig a segment of the small intestine was isolated and suspended in a bath.

1. Smooth muscle of the stomach.

In cats under chloralose-urethane the blood of the spleen was discharged by intravenous injection of 50 μg adrenaline, the splenic vessels tied, and the spleen removed. A small glass cannula was inserted in the splenic artery, with the tip pointing towards the coeliac artery, which could intermittently be obstructed by pulling a ligature during the application of substances. In another set of experiments the cannula was inserted in a gastric branch of the coeliac artery.

Motor activity is measured by introducing a rubber balloon through the oesophagus into the stomach, and its changes in pressure are transmitted to a U-shaped manometer, filled with bromoform. The pressure changes are recorded by a smoke-drum kymograph. The action of the phosphate compounds is compared with the effect of vagal stimulation, acetylcholine and histamine[1].

2. Smooth muscle of the bladder.

In cats under chloralose-urethane a cannula is inserted in the sacral artery immediately caudal to the rise of the iliac arteries. The two hypogastric and the caudal part of the sacral arteries are tied; the umbilic arteries from which the four vesical arteries arise are now the only open channels. After removal of the symphysis a glass tube is introduced into the bladder through the urethra. In a number of experiments the ureters are ligated. The empty bladder is filled with 10—20 ml warm Tyrode solution and the pressure changes are transmitted to a manometer of the same type as used in recording stomach motility. Also here the actions of the phosphorus compounds are compared with the reaction to acetylcholine and histamine. In cats weighing 2.8—3.2 kg the weight of the bladder is approximately 5—6 g.

For both stomach and bladder the volume applied intra-arterially is 0.7 ml, 0.5 ml Tyrode being injected immediately after application of the substances. In each animal repeated *controls* are performed by injection of 0.7 ml Tyrode solution. The time used for injection of the different substances is approximately equal.

In bladder and stomach the effect of the different substances is investigated before and after *atropinization*. Atropine sulphate is given

[1] The histamine doses are given in terms of histamine diphosphate.

intra-arterially in quantities of 0.3—1.0 mg, a dose which abolishes or greatly reduces the response to maximal electric vagal stimulation and to 20—50 μg acetylcholine, intra-arterially injected.

3. Small intestine of the guinea-pig.

The motor activity of the longitudinal muscles is recorded by immersing a segment of the ileus, 20—25 mm in length, in a bath containing 3 ml oxygenated Tyrode at 36° C. The free end of the gut is attached to a frontal lever, and its movements are registered on a smoked drum. The substances were tested before and after atropinization (atropine sulphate 1: 200,000 in the bath).

The various phosphates are applied iso-osmotically by replacing part of the NaCl + water in a Tyrode solution by an equivalent amount of the staple phosphate solution in question. The further dilution is made by adding Tyrode solution with pH 7.3 and a temperature of 37° C. The preparation of the phosphorus compounds is described in a previous paper (BUCHTHAL et al. 1944). The substances were supplied by the kindness of Dr. A. DEUTSCH (Research Laboratory, A. B. Leo, Hälsingborg).

Results.

1. Stomach and bladder of the cat.

On the smooth muscle of these two organs the substances investigated exert qualitatively identical effects and they will be dealt with together.

Na-ATP in concentrations of 0.24—11.6 $\times 10^{-6}$ mol/ml (= 0.034 —1.6 $\times 10^{-6}$ mol/g muscle[1]) releases contractions immediately after the injection, as does acetylcholine (Fig. 1). The tension develops steeply, and its duration is about the same as after injection of acetylcholine. Fig. 2 shows the effect of a dose slightly above threshold (0.017 mg/g muscle = 0.035 $\times 10^{-6}$ mol/g muscle) preceded by approximately the double concentration. From this figure it is seen that there exists a definite relation between dose and effect, the concentration-action curve being rather steep. With larger doses the duration of the contraction is greatly prolonged. In a fresh preparation repeated injection of ATP (4—5 times) evokes approximately equal tension, while the response decreases after a longer series of injections.

As compared with acetylcholine and histamine, 3.9 $\times 10^{-6}$ mol/ml Na-ATP causes an effect on the stomach which corre-

[1] The figures given per g muscle refer to the bladder.

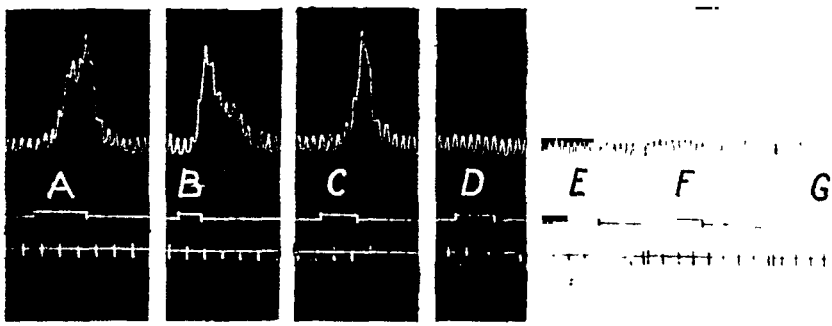

Fig. 1. Motor activity of the stomach of the cat after

A. vagal stimulation of 30 sec duration.
B. intra-arterial injection of 10 μg acetylcholine.
C. » » » » 0.27×10^{-6} mol adenosine triphosphate.[1]
D. » » » » 0.7 ml Tyrode's solution.
C. » » » » 5×10^{-6} mol orthophosphate.
F. » » » » 4.1×10^{-6} mol tripolyphosphate.
G. » » » » 4.9×10^{-6} mol pyrophosphate.

Time marks: 10 sec.
The signal above time marks denotes intra-arterial injection.

sponds to the response after application of 12 μg acetylcholine or 15μg histamine; on the *bladder* 0.13 mg/g muscle Na-ATP (1.8×10^{-6} mol/ml $= 0.25 \times 10^{-6}$ mol/g muscle) releases an effect simular to that of 2.5 μg acetylcholine or 20 μg histamine.

Fig. 2. Application of Na-adenosine triphosphate to the bladder of the cat.

A. 30 μg/g muscle $= 0.34 \times 10^{-6}$ mol.
B. 17 μg/g muscle $= 0.17 \times 10^{-6}$ mol.

Time marks: 10 sec.

Atropinization to a degree which nullifies or greatly diminishes the responses to 20—50 μg acetylcholine, does not abolish the effect of ATP (Fig. 6 C). In some experiments the effect of ATP, like that of histamine, is slightly reduced by atropinization.

Although in some instances an inhibition of the automatic rhythmic contractions may occur after application of ATP, *we have never observed a decrease in tension of smooth muscle* with this or the other substances investigated. ATP does not potentiate the effect of acetylcholine as observed in skeletal muscle (BUCHTHAL and KAHLSON 1944).

As differences in the effect of different salts of adenosine triphosphoric acid on flow-bi-

[1] Here and in figures 2—6 the phosphorus compounds are calculated as the total injected amount in moles, while otherwise the doses are defined by mol/ml and mol/g muscle.

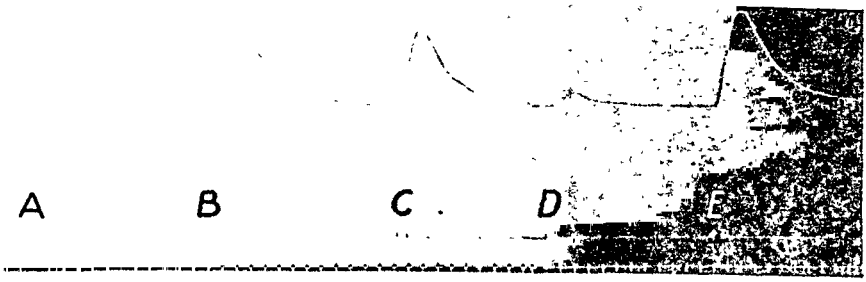

Fig. 3. Reaction of the bladder to:

A. 0.78×10^{-6} mol sodium adenosine triphosphate
B. 0.78×10^{-6} mol lithium » »
C. 0.78×10^{-6} mol potassium » »
D. 0.78×10^{-6} mol sodium » »
E. 0.78×10^{-6} mol lithium » »

Time marks: 10 sec.

refringence and viscosity of myosin solutions have been described by NEEDHAM et al. (1941) it seemed of interest to compare their effect on living muscle. Sodium ATP, potassium ATP and lithium ATP were tested in concentrations of $0.3—11.6 \times 10^{-6}$ mol/ml ($= 0.04—1.6 \times 10^{-6}$ mol/g muscle). Although NEEDHAM et al. report a tenfold stronger effect of K-ATP than of Na-ATP, on myosin, there is no significant difference in the effect of Na, K, and Li-ATP on mammalian smooth muscle. K-ATP depresses, however, the reaction of subsequently applied Na-ATP (Fig. 3). An iso-osmotic Tyrode-solution with excess of potassium ions in the same amount as applied with K-ATP has no such inhibiting effect. It is interesting that K-ATP does not diminish the response to subsequent injection of Li-ATP.

Apart from ATP, the following other phosphates were tested on the smooth muscle of stomach and bladder and found ineffective:

Adenosine diphosphate in amounts of 2.5×10^{-6} mol/ml ($= 0.35 \times 10^{-6}$ mol/g muscle, Fig. 4);

Adenosine diphosphate + orthophosphate in a concentration of $(5.0 + 5.0) \times 10^{-6}$ mol/ml ($= (0.7 + 0.7) \times 10^{-6}$ mol/g muscle, Fig. 4);

Adenylic acid ($0.4—7.8 \times 10^{-6}$ mol/ml $= 0.06 — 1.0 \times 10^{-6}$ mol/g muscle) and adenylic acid + pyrophosphate ($(1.56 + 1.56)$ to $(2.5 + 2.5) \times 10^{-6}$ mol/ml $= (0.1 + 0.1)$ to $(0.2 + 0.2) \times 10^{-6}$ mol/g muscle, Fig. 5);

Pyrophosphate (7×10^{-6} mol/ml $= 1.0 \times 10^{-6}$ mol/g muscle,

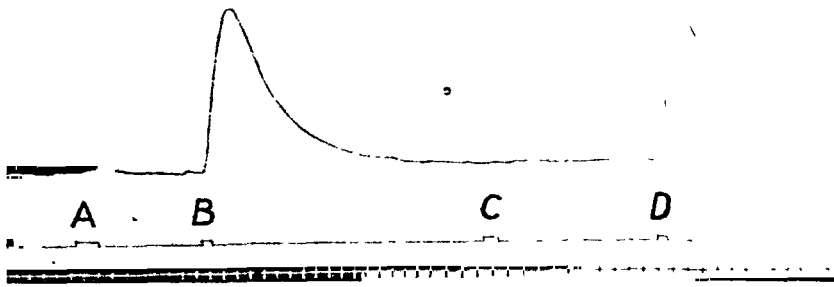

Fig. 4. Effect of adenosine diphosphate, adenosine diphosphate + orthophosphate and adenosine triphosphate on the bladder of the cat.

A. $(0.84 + 0.84) \times 10^{-6}$ mol adenosine diphosphate + orthophosphate.
B. 0.78×10^{-6} mol adenosine triphosphate.
C. 1.74×10^{-6} mol adenosine diphosphate.
D. 0.78×10^{-6} mol adenosine triphosphate.

Time marks: 10 sec.

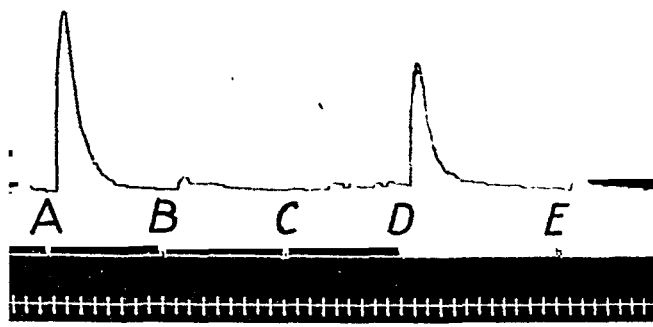

Fig. 5. Application of adenylic acid and adenylic acid + pyrophosphate to the bladder of the cat.

A. 0.68×10^{-6} mol adenosine triphosphate.
B. $(1.1 + 1.1) \times 10^{-6}$ mol adenylic acid + pyrophosphate.
C. 2.2×10^{-6} mol adenylic acid.
D. 0.68×10^{-6} mol adenosine triphosphate.
E. $(1.73 + 1.73) \times 10^{-6}$ mol adenylic acid + pyrophosphate.

Time marks: 10 sec.

Figures 6 and 1) and orthophosphate (14×10^{-6} mol/ml $= 2.0 \times 10^{-6}$ mol/g muscle, Figures 6 and 1).

The effect of sodium tripolyphosphate (6×10^{-6} mol/ml $= 0.83 \times 10^{-6}$ mol/g muscle) is either absent (Fig. 1) or slight.

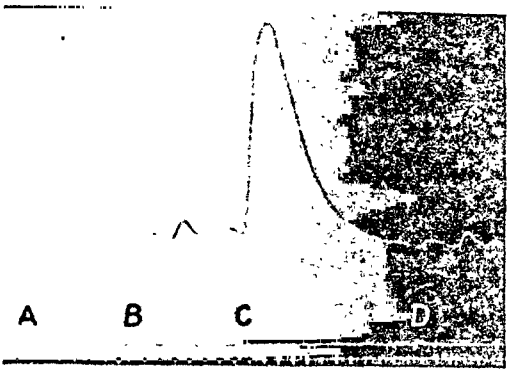

Fig. 6. Application of pyrophosphate, orthophosphate and adenosine triphosphate [illegible] bladder of the cat.

A. 4.9 × 10^{-6} mol pyrophosphate
B. 5.0 × 10^{-6} mol orthophosphate
C. 5.4 × 10^{-6} mol adenosine triphosphate
D. 20 μg acetylcholine.

Time marks: 10 sec.

2. The small intestine of the guinea-pig.

Fig. 7 shows that Na-ATP (B, C) in a concentration of 0.91 ×10^{-6} mol/ml in the surrounding Tyrode-bath causes a contraction of about the same size as 3.3 × 10^{-6} μg acetylcholine (A) and 2.5 ×10^{-2} μg histamine (D). Na-, K- and Li-ATP have identical reactions and release contraction already in concentrations of 0.015 × 10^{-6} mol/ml (= 22 μg) in the bath. Atropinization does not affect

Fig. 7. Small intestine of the guinea-pig.

A. acetylcholine 1.1 ×10^{-6} μg/ml.
B. adenosine triphosphate 0.91 × ×10^{-6} mol/ml.
C. adenosine triphosphate 0.91 × ×10^{-6} mol/ml.
D. histamine 0.83 ×10^{-2} μg/ml.

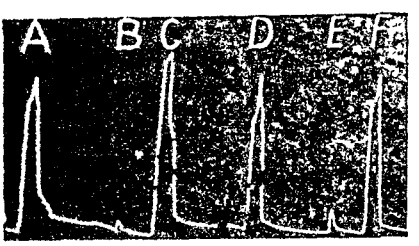

Fig. 8. Small intestine of the guinea-pig.

A. histamine 0.67 × 10^{-2} μg/ml.
B. adenylic acid + pyrophosphate (1.28 + 1.28) × 10^{-6} mol/ml.
C. histamine 0.67 × 10^{-2} μg/ml.
D. » 0.67 × 10^{-2} μg/ml.
E. adenylic acid 1.8 × 10^{-6} mol/ml.
F. histamine 0.67 × 10^{-2} μg/ml.

the response to ATP. The other phosphates tested (Na-tripolyphosphate 0.67×10^{-6} mol/ml, Na-orthophosphate 0.41×10^{-6} mol/ml) were ineffective, just as is the case with the stomach and the bladder of the cat. Fig. 8 demonstrates the insensitiveness of the gut to adenylic acid (1.8×10^{-6} mol/ml = 1.9 mg/3 ml) and adenylic acid + pyrophosphate ($(1.28 + 1.28) \times 10^{-6}$ mol/ml = 1.33 + 1.68 mg/3 ml). Sodium pyrophosphate represents, however, an exception and evokes contraction in the longitudinal muscle of the gut in concentrations of $0.075 - 0.225 \times 10^{-6}$ mol/ml. This effect was, however, not regularly present. The action of ADP and ADP + orthophosphate was not investigated.

In no experiment and with no concentration employed was an inhibitory or a relaxing effect of the different substances observed.

Discussion.

In the specimens of smooth muscle we have investigated, ATP evokes contraction in extremely small amounts. This finding provides further bearing on the assumption that the substance is engaged as a physiological agent in the release of contraction. One may suggest that in the humoral mechanism involved in the initiation of contraction ATP represents a further link, the substance being activated by a preceding liberation of acetylcholine.

The data hitherto presented as to the action of ATP and other adenylic compounds are rather disjointed. On the isolated uterus of the guinea-pig ZIPF (1931), BENNET and DRURY (1931), EULER and GADDUM (1931), DEUTICKE (1932) and GILLESPIE (1934) find motor stimulating effects of adenosine, adenylic acid, adenosine triphosphate and inosine triphosphate. On the small intestine of the rabbit and the cat DRURY and SZENT-GYÖRGYI (1929), EULER, EULER and SCHENK (1940), ABDON (1942) et al. find a decrease in tension and an inhibition of rhythmic activity after application of adenosine, adenylic acid, cozymase and adenosine triphosphate. GILLESPIE (1934) reports an initial decrease followed by an increase in tension after addition of ATP.

In our experiments we have never observed decrease in tension caused by ATP or other phosphorus compounds, although a slight inhibition of rhythmic activity may occur simultaneously with the rise in tension when ATP is applied. It is of interest to note that in contrast to our findings on skeletal muscle, the specimens of smooth muscle investigated here react highly speci-

fically to ATP, adenosine diphosphate, adenylic acid and other phosphates being without effect. Even application of adenosine diphosphate + orthophosphate is ineffective. Thus smooth muscle reacts just as specifically towards ATP as does thrice precipitated myosin in NEEDHAM et al.'s experiments (1942). Adenosine diphosphate which interacts with relatively unpurified myosin has no effect on smooth muscle.

Added in the proof: We have lately examined the stimulating effect of *inosine triphosphate* on the smooth muscle of the bladder. Compared with ATP inosine triphosphate is less active, the threshold dose being approximately 5 times higher than that of ATP.

Summary.

1. With the following substances investigated for their action on smooth muscle by close arterial injection in the stomach and the bladder of the cat it was demonstrated that: Sodium-, potassium-, and lithium adenosine triphosphate release contraction, while adenosine diphosphate, adenosine diphosphate + orthophosphate, adenylic acid, adenylic acid + pyrophosphate, inorganic tripolyphosphate, pyrophosphate and orthophosphate applied iso-osmotically as sodium salts at pH 7.3 are ineffective.

2. Potassium adenosine triphosphate depresses the reaction of the smooth muscle to subsequent injections of sodium adenosine triphosphate, while the action of lithium adenosine triphosphate remains uninfluenced.

3. The isolated small intestine of the guinea-pig contracts after application of the different salts of adenosine triphosphate, while the other phosphates investigated are without effect. Pyrophosphate makes an exception and may cause contraction.

4. None of the phosphorus compounds investigated exerts a relaxing effect on the stomach and bladder of the cat or on the small intestine of the guinea-pig.

5. The experiments presented in the present and the preceding papers suggest that adenosine triphosphate acts as a further link in the humoral mechanism involved in the release of contraction, the substance possibly being activated by the liberation of acetylcholine.

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On the Alleged Existence of the Enzyme Glycocyamase.

By

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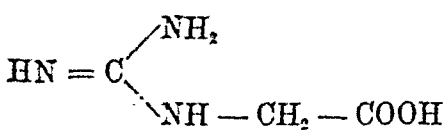
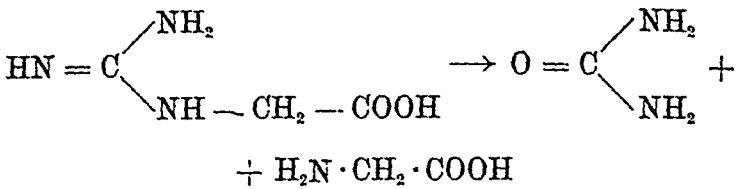
Among the numerous guanidino compounds found in animal organisms, guanidino acetic acid, or glycocyamine as it is often and more conveniently called, stands out as one of particular interest to biochemists. Its structural formula



brings clearly out its close relationship to creatine. In fact, glycocyamine is transformed into creatine by a simple methylation, and from the experimental evidence already available the compound can probably be regarded as one of the mother substances of creatine in the animal body (see for instance GUGGENHEIM, 1940).

On account of this it is clear that enzymes which attack or transform glycocyamine must be of considerable biological importance. For this reason, and also for the intrinsic interest that always attaches to new enzymes from the purely chemical point of view, they deserve the attention of biochemists. Now KARASHIMA (1928) has found, in contradiction to previous workers, that the liver of mammals contains a specific enzyme capable of hydrolysing glycocyamine to urea and glycine in the following way:



This contention of KARASHIMA has found some support a few years ago in the work of FELIX and SCHNEIDER (1938). They found that the hydrolysis of glycocyamine by an enzyme preparation from liver is unaffected by the presence of manganese ions. Manganese, however, is known as a strong activator of arginase, and FELIX and SCHNEIDER therefore conclude that the latter enzyme is not responsible for the observed hydrolysis.

Apart from this, however, no one seems to have worked experimentally on the question. This is rather deplorable, since at the present time there is a considerable divergence of opinion in the literature as to the interpretation of the results just quoted. Thus OPPENHEIMER (1938) does not in any way seem ready to accept as conclusive the present evidence for the existence of glycocyamase. He suggests that a reinvestigation should be carried out with purified arginase solutions. KRAUT and KOFRANYI (1941) state quite bluntly and, as far as the present writer can see, without reference to further experimental work, that glycocyamine is hydrolysed by arginase. In view of this rather glaring disagreement it was thought worth while to attempt a reinvestigation of the problem, and the present paper is a report on the results.

Before entering into experimental details it may not be out of place to discuss briefly certain general features of the problem under investigation. The problem of proving the identity of two enzymes is, in the present state of development of enzymic chemistry, in many cases a rather difficult one. This is primarily due to the fact that we are dealing with often very crude enzyme solutions, instead of with crystalline substances. However, progress towards the solution of the identity problem is still possible by examining the kinetics of the enzymic effects (for instance the pH dependence) and the influence of various activators and inhibitors. This is the method which we attempt to apply in the present piece of work. This appears justifiable, since it is at the present stage of development impossible to obtain really purified arginase solutions; it is therefore likewise

impossible at the present time to follow up the suggestion of OPPENHEIMER (see above).

Furthermore, from a consideration of the substrate specificity of arginase it seems possible to derive some information of interest for our particular problem. A considerable amount of work has been done on the substrate specificity of arginase (see for instance KRAUT and KOFRANYI, 1941). Thus it has been found that carbamino arginine is hydrolysed by arginase to carbamino acid and ornithine. Hence the guanidino group in arginine can be replaced by the carbamino group without affecting the hydrolysability; the reaction velocity, however, may well be changed. Other alterations in this group, such as nitration, methylation and benzoylation, lead to nonhydrolysable substances. The presence of the α-amino group is not decisive, since methyl arginine and arginyl arginine are both hydrolysable, the latter compound, however, only at that part of the molecule which carries the free carboxylic group. The α-amino group can also be entirely absent, the compound still retaining its hydrolysability. This relative unimportance of the amino group is also brought out by the fact that if it is replaced by alanine, as in octopine, the new substance is still attacked by arginase. We may also mention that α-oxy δ-guanidino valeric acid, α-guanidino valeric acid and α-amino—δ-guanidino capronic acid are all hydrolysed by the enzyme. Hence the length of the carbon chain is relatively unimportant. Finally we mention that a free carboxylic group has been found necessary. This was already indicated above, and is further corroborated by the fact that esters of arginine are not hydrolysed by arginase.

After surveying this evidence the reader will probably agree that there is not much in it that would seem to necessitate the assumption of a specific glycocyamase. This may also be the reason why some authors, as mentioned above, have been unwilling to accept as conclusive the evidence furnished up to now for the existence of a specific enzyme for the hydrolysis of glycocyamine.

Experimental Part.

Enzyme solution. All the work was done with a glycerol extract from pig's liver. It was prepared by cutting the liver into pieces and grinding it carefully with sea sand in a mortar. It was then extracted with glycerol for a few hours at room temperature and over night in the

ice box. The next day it was filtered or centrifuged, and used immediately. No extract was used which was older than three or four days.

Substrate. Glycocyamine was prepared by the method of NENCKI and SIEBER (1878), which works very smoothly and with satisfactory yield.

Method of analysis. To follow the enzymic hydrolysis we have applied the urease method for determining the amount of urea set free in the process. A very convenient modification of this method has been worked out by KREBS and HENSELEIT (1932), and this has been applied in the present investigation. The principle of their procedure is to measure manometrically in a Warburg apparatus the amount of carbon dioxide set free by the enzymic hydrolysis of urea. The technique is as follows:

Reagents:

1. Acetate buffer: 27.2 g crystalline sodium acetate and 6 g glacial acetic acid are filled up with water to the mark in a 100 ml flask. pH is around 5.

2. Urease solution.

Procedure: The main chamber of a Warburg vessel was filled with 2 ml of the solution to be analysed and with 0.3 ml of the acetate buffer solution. These proportions have always proved sufficient for obtaining the correct acid reaction, with the concentrations used in ordinary buffer solutions. The side vessel contained 0.3 ml urease solution. After temperature equilibrium had been reached (10—15 minutes) the urease solution was introduced into the main chamber. The final manometer readings were usually taken after 20—30 minutes.

Buffer solutions. The experiments were carried out in glycine buffers (4 ml m/10 NaOH plus 6 ml m/10 glycine). As far as is known the activity of arginase is almost the same in glycine, phosphate and veronal buffers. When phosphate is present the urea determinations by the KREBS-HENSELEIT method requires longer time than with the other buffers, and since the glycine buffers were immediately at hand, all the work was carried out with them.

Experimental procedure and results: Glycocyamine is only sparingly soluble in water and ordinary buffer solutions in the physiological pH range. It is therefore not possible to work with higher concentrations of the substrate. Hence our experiments were carried out with a glycocyamine solution prepared by shaking a suitable amount of glycine buffer with a surplus of glycocyamine for about 1 hour.

To 10 ml of this glycocyamine solution were added 5 ml of the enzymic extract described above, and a suitable amount (see below) of the heavy metal salt, or other compound, the influence of which on the hydrolysis was to be examined. After a suitable time the enzyme was inactivated by placing the flasks for a short time in hot water, and their contents analysed for urea immediately afterwards. Experiments without addition of any activator or inhibitor were al-

ways carried out at the same time, and likewise blank experiments to determine the amount of urea in the enzyme solution. The latter determinations were made by adding 5 ml enzymic extract to 10 ml glycocyamine solution, immediately inactivating the enzyme and analysing for urea in the way described above.

An important point must be discussed here. In applying the urease method it is essential to remember that this enzyme is strongly inactivated by the presence of heavy metal ions. Fortunately, however, it has been shown by HELLERMAN and PERKINS (1935) that this difficulty can be easily overcome by adding a suitable amount of cyanide to the solution to be analysed before the urease is added. This was done throughout the present work, and care was taken to follow the instructions of HELLERMAN and PERKINS.

The experiments were all performed at 25° ± 0.1, and the enzymic hydrolysis lasted for 24 hours ± 5 minutes. Double experiments and double analyses were carried out throughout the whole work.

What is actually measured in these experiments is in each case the amount of urea set free in the hydrolytic process during a fixed interval of time. We shall regard this quantity as a measure of the enzymic activity, and even, as a convenient abbreviation, term it enzymic activity. We shall further express the increase in enzymic activity on addition of salts in per cent of the activity without salt. This increase in activity is the only quantity of interest in the activation experiments, and by restricting us to it in what follows we avoid burdening the reader with unnecessary and tedious numerical details. A detailed description of the numerous individual experiments underlying the present report will therefore not be attempted.

In conformity with this we quote in Table 1 the results obtained for one particular enzymic solution. The first column

Table 1.

	Concentration	Activation
Mangano sulphate	m/460	280 per cent
» nitrate	m/500	250 »
» chloride	m/400	265 »
Cadmium sulphate	m/800	300 »
» nitrate	m/900	300 »
» chloride	m/600	290 »
Cobalto nitrate	m/900	290 »
Nickel nitrate	m/900	90 »
» chloride	m/800	100 »
Cupri chloride	m/500	50 per cent inhibition
» sulphate	m/500	55 »
Potassium cyanide	m/200	No effect

specifies the salt the effect of which was investigated, the second column gives the molarity in which it was present in the reaction mixture and the last one gives the activation or inhibition, expressed in the way defined above.

Similar experiments were carried out with three other enzyme solutions, obtained from different animals in the way described above. The results were qualitatively exactly the same, and further details may therefore be omitted.

Finally some experiments were carried out in order to determine approximately the pH optimum for the hydrolysis of glycocyamine. The work was carried out using glycine buffers and proceeding as above. For all four enzyme solutions a maximum was found to lie somewhere in the range 8.6—9.2. Apart from the statement of this result, the reproduction of the curves obtained is probably of no great interest and is therefore omitted.

Discussion.

From the literature (Bamann and Myrbäck 1941, Oppenheimer 1936—1938, Kraut and Kofranyi 1941) it appears to be well established that arginase is strongly activated by manganese, cobalto, cadmium and nickel ions. In concentrations of the order of about m/1,000 their effects are of the same order of magnitude. It is further reported that cyanide shows no effect in the alkaline range. Copper is stated to be a strong inhibitor of arginase. In surveying the experimental results reported in the previous section the reader will probably agree that they fit in rather nicely in this picture. This conclusion is corroborated by our results for the pH optimum, which likewise agree well with those of other workers.

On correlating these findings with what was said above about the substrate specificity of arginase, we may conclude that, as far as the present work indicates, there are no grounds for assuming the existence of a specific glycocyamase.

It is unfortunately difficult to advance any definite explanation of the diverging results in the literature. It may, however, not be entirely out of place to draw attention to the circumstance, that frequently organs have been found to give either completely inactive or only feebly active arginase solutions, but on addition of manganese salts they have become active. It may be that this

point has not always been properly appreciated, and if so, it may at least account for those experiments in which glycocyamine was found not to be hydrolysed by arginase.

The author takes great pleasure in expressing his best thanks to Prof. EGE, in whose institute this piece of work was carried out, for his generous hospitality.

Summary.

It is found that glycocyamine is hydrolysed by a glycerol extract from pig's liver. From activation experiments it is concluded that the enzyme responsible for the reaction is probably arginase, and not a specific glycocyamase.

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On the Distribution of Asparaginase.

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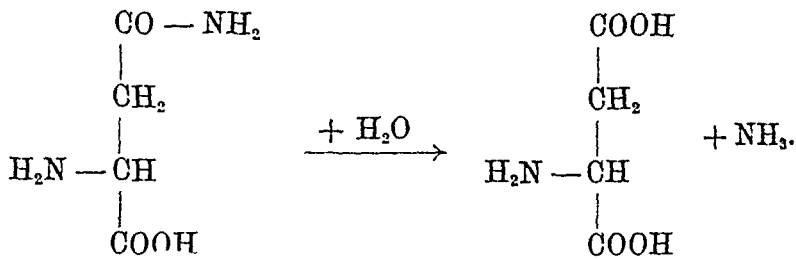
Asparaginase is a well known enzyme which belongs to the group of amidases and catalyses the hydrolytic cleavage of asparagine to ammonia and aspartic acid, i. e. the reaction



This enzyme is one of very remarkable specificity. At least as far as is known at present, asparagine seems to be the only substrate which is attacked by it. This narrow specificity property may indicate that the enzyme has important biological functions to fulfill. On account of this circumstance a fairly large amount of work has been expended in the study of this enzyme, and as a consequence an extensive literature has grown up in this field of enzymic research. However, several problems still remain unsolved, and one of these will occupy us in the present note. It concerns the distribution of the enzyme in certain animal organisms, which, to judge from the existing literature, offers some rather striking peculiarities. It has been pointed out by several authors that the enzyme can be expected to occur in the organs of all higher animals, since it can probably be assumed to form a normal chain in the breakdown of proteins.

However, CLEMENTI (1926) reached the surprising conclusion that asparaginase is to be found only in birds, herbivora and omnivora, and is missing in cold-blooded animals and in invertebrates. He advocates the view that the enzyme is to be regarded as an adaptation on the part of certain organisms to asparagine-containing food. A part of this statement has already been disproved by other workers, who found the enzyme in other warm-blooded animals than those specified by CLEMENTI (see OPPENHEIMER (1936—38), KRAUT and KOFRANYI (1941). The necessity of verifying his statement also for the cold-blooded animals has already been pointed out by other students of the subject (see OPPENHEIMER (1936—38), KRAUT and KOFRANYI (1941). In accordance with this the present writer some time ago occupied himself with this problem, and the present note is a short report on his results.

Experimental Part.

Substrate. The asparagine used in the experiments to be described below was a commercial product, which was carefully purified by recrystallisation. Its physical properties agreed closely with those given for asparagine in the standard chemical literature.

Enzyme solution. A purification of asparaginase has not yet been carried through successfully. One of the reasons for this is probably the instability of the enzyme, in particular the fact that it is easily destroyed by acid, and also by certain organic solvents and precipitation reagents such as acetone. We are therefore compelled to work with rather crude enzyme solutions. In the literature favourable results have been reported in some cases with aqueous glycerol as a solvent. Thus, SCHMALFUSS and MOTHES (1930) were able to study the asparaginase of Aspergillus niger by preparing aqueous glycerol extracts of that mould, though the solutions thus obtained were only rather weakly active. SUZUKI (1936) obtained active asparaginase solutions both with glycerol and with a 10 percent saccharose solution. He found aqueous solutions to be only feebly active. So far, all asparaginase solutions described have been rather unstable, their activity diminishing greatly within a few days. They must therefore be used at once.

For the purpose of the present work aqueous glycerol and 10 percent saccharose solutions were used to obtain active enzyme preparations.

Method of analysis. To follow the course of the enzymic reaction, two methods have been applied in the past. The first one consists in the determination of the ammonia liberated in the process, for instance by applying the procedure developed by PARNAS and HELLER

(1924) for the determination of ammonia in blood. The second method depends on the determination by titration in alcoholic solution with phenolphtalein as indicator according to WILLSTÄTTER and WALDSCHMIDT-LEITZ (1921) of the amount of carboxylic groups liberated during the enzymic reaction by the breaking of the acid amide bond. This latter method has been applied in the present work. It is so simple and has been described so many times in standard handbooks that details of the procedure may be omitted here.

Experimental results. Our experiments were carried out with extracts from the livers of some cold-blooded animals.

In the first place frogs (*Rana esculenta L.*) were used. The livers from six freshly killed animals were carefully ground in a mortar with sea sand, and the mixture then divided into two portions. One of these was extracted with 30 ml of 80 percent aqueous glycerol, and the other portion with 30 ml of a 10 percent saccharose solution. The extraction lasted about 3 to 4 hours at room temperature, the mixture being stirred now and then, and finally it was left standing in the ice box overnight. It was then spun down in the centrifuge. 10 ml of the enzyme solution thus obtained were added to ca. 40 ml of phosphate buffer (pH around 7.5—7.7, which agrees well with the pH optimae of asparaginase found by other workers), in which a suitable amount of asparagine was dissolved. The exact concentration of the amino acid was not determined. However, for the purpose in hand this appeared unnecessary, since what is really of interest in our experiments is the number of carboxylic groups as determined by the WILLSTÄTTER and WALDSCHMIDT-LEITZ titration and given by the amount of n/20 alcoholic potassium hydroxide required by a certain amount, in our case 3 ml, of the reaction mixture. It should be born in mind that this quantity includes also the carboxylic groups in the enzyme solution. However, this is fortunately rather irrelevant, for in the end we are interested only in the *changes* in the amount of carboxylic groups in the reaction mixture, and blank experiments prove that the amount of carboxylic groups in the enzyme solution remains constant, at any rate in the interval of time required by our experiments. We believe, therefore, that we are entitled to regard the observed changes in the titration numbers as directly measuring the hydrolytic cleavage of asparagine. It is clear that allowance must be made for selfhydrolysis of the amino acid, and as will appear below this was always done in our experiments.

The reaction mixture was left to stand at 24° for a suitable time. At the beginning and at the end of this time interval 3 ml of the mixture were pipetted off for the WILLSTÄTTER and WALDSCHMIDT-LEITZ titrations. Double experiments and sometimes also double titrations were carried out. In the latter cases 6 ml of the reaction mixture were pipetted off each time. Blank experiments were always carried out in order to account for the selfhydrolysis of the amino acid, as well as for any change in the amount of carboxylic groups contained in the enzyme solution. Both these corrections proved to be very small, and the latter in fact practically zero; they are included in the results given below.

The results are given in table 1.

Table 1.

Consumption in ml of N/20 alcoholic KOH.

Animal	Glycerol extracts		Extracts with 10 percents accharose solution	
	At the start	Second determination[1]	At the start	Second determination[1]
Frog	5.9	5.9 (18)	5.5	5.5 (18)
	6.1	6.1 (18)	5.6	5.6 (18)
Trout	3.4	3.9 (22)	3.4	4.1 (22)
	3.4	3.8 (12)	3.4	4.2 (22)
Plaice	5.7	6.3 (24)	5.6	7.0 (24)
	5.7	6.4 (24)	5.6	7.1 (24)

In order to check these results titrations were also carried out using the modification of the WILLSTÄTTER and WALDSCHMIDT-LEITZ method described by GRASSMANN and HEYDE (1929). The colour change of the indicator used here (thymol phtalein) is not easy to observe sharply, but the difficulty can be satisfactorily overcome by using a suitable colour standard. The results were quite similar to those given above, and need therefore not be reproduced here.

Three other sets of experiments similar to the one described above were carried out on other animals. In two of these the duration of the experiment was extended to 40 hours. The qualitative results were exactly the same, and numerical details are therefore omitted.

[1] The figure in brackets gives the number of hours after the first determination.

The next experiments were carried out on livers from the trout (*Salmo irideus* Gibb.). The organs were excised from three freshly killed animals and treated as described above for frog livers: careful grinding with sea sand, and then extraction of half of the thick liver paste with ca. 30 ml aqueous glycerol, and of the other half with ca. 30 ml of a 10 percent saccharose solution. The rest of the work, including titrations and blank experiments, were carried through exactly as before (table 1.) Measurements by the procedure of GRASSMANN and HEYDE gave similar results.

Three other sets of experiments on trout livers were carried out in the same way. In two of these sets the hydrolysis was followed for 38 hours. Qualitatively similar results were obtained in all cases, and numerical details are therefore omitted. We may perhaps mention that the saccharose solutions always proved to be considerably more active than the aqueous glycerol extracts.

In a final series of experiments the liver of the plaice (*Pleuronectes platesa* L.) was investigated. The livers from four freshly killed animals were excised and lumped together. They were treated exactly as described above for the other animals, and the whole rest of the work, including the titrations and blank experiments, were carried through as before. The GRASSMANN-HEYDE procedure gave essentially the same results as the titrations according to the method of WILLSTÄTTER and WALDSCHMIDT-LEITZ. In the whole four sets of experiments were carried through with the same qualitative results in all cases. In particular the saccharose solution again appeared to be a more efficient means for extracting the enzyme than aqueous glycerol. To save space we quote only the full results of one set of experiments (table 1).

Discussion.

The results reported above show clearly that liver extracts from the trout and the plaice are capable of hydrolysing asparagine. We consider this to prove the occurrence of asparaginase in these organisms. The case of the frog is not so straightforward. The liver extracts did not show the slightest sign of enzymic activity, and must therefore be considered as free of asparaginase. However, the enzyme content of the liver may possibly

depend on such factors as for instance the season, and the state of nutrition and general health of the animals, and so on. The frogs used in our experiments had been kept in captivity for quite a while, and under such conditions they often become rather undernourished. It is unknown how far this may affect the enzyme content of the liver. Though otherwise the animals appeared to be in perfect health, the most cautious interpretation of the experiments probably is that frog's liver contains only a very small amount of asparaginase. We see therefore that although the distribution of the enzyme may show great irregularities from one cold-blooded animal to another, it is not missing in this group of animals.

The present work was carried out in the institute of Prof. EGE, to whom the author wishes to express his warmest thanks for generous hospitality and support.

Summary.

It is found that asparaginase occurs in the livers of certain cold-blooded animals. The method used consists in preparing enzyme extracts with aqueous glycerol and saccharose solutions as solvents, and in following the hydrolytic cleavage of asparagine during the enzymic reaction by means of the WILLSTÄTTER and WALDSCHMIDT-LEITZ method of titration in alcoholic solution. The work of CLEMENTI could not be supported.

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Studies on the Liberation of Renin in completely Ischemic Kidneys.

By

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Introduction.

In 1898 TIGERSTEDT and BERGMAN, in a publication from this Institute, showed that in the cortex of normal kidneys there is a pressor substance, which they termed "renin", and which they supposed might be the cause of hypertension in renal diseases. The possible importance of this renal pressor substance for the pathogenesis of hypertension was not, however, generally recognized until GOLDBLATT and collaborators in 1934 had begun to publish their investigations on experimentally induced hypertension. GOLDBLATT, as we know, induces hypertension in various test animals by producing a partial ischemia of one or both kidneys by the occlusion of the renal arteries with silver clamps which can be screwed up more or less tightly, so that the degree of hypertension can be graduated. With this method a number of valuable investigations into renal hypertension have been carried out in the course of the last ten years.

That a pressor substance is formed also in the totally ischemic kidney was first shown by DICKER (1937), who found, in experiments on dogs, that the reestablishment of the circulation, after occlusion of the renal artery for 24 hours, was followed by a rise of blood pressure. The rise was to the extent of 40—50 mm Hg and proceeded for 20—30 minutes. It could also be elicited by the intravenous injection of a perfusate of the ischemic kidney into the femoral vein, whereas injection into a mesenterial vein produced no effect.

Investigations into the effect of total ischemia of shorter duration have been made by TAQUINI (1940), who occluded the renal pedicle, or only the renal artery, of dogs and found a manifest elevation of their blood pressure when the clamps were removed after the lapse of $5^1/_2$—$6^1/_2$ hours. That this effect was humoral and not reflex was indicated by the following observations. Firstly, the effect failed to manifest itself when the circulation in the kidney had been arrested either owing to thrombosis in the renal vessels or because the arteries, besides being clamped, had also been ligated. Secondly, a kidney which had been totally ischemic for 5—7 hours showed a distinct effect on transplantation to the neck of another dog. Finally, in a case where there were two arteries to one kidney, and where only one of them was clamped, no formation of pressor substance was observed in that kidney. TAQUINI also showed the presence of pressor substance in renal venous blood after total ischemia both on transfusion of the blood to normal dogs and in experiments with the perfusion of isolated dog extremities or the Löwen-Trendelenburg preparation.

Investigations into the formation of pressor substance in totally ischemic kidneys were subsequently taken up by COLLINS and HAMILTON (1940). In experiments with the occlusion, for $5^1/_2$—$6^1/_2$ hours, of the renal pedicles of dogs, they confirmed the findings of previous investigators, in that the release of the clamps was followed by elevations of blood pressure averaging 35 mm. Occlusion of shorter duration resulted in a lesser rise, which, however, was already perceptible after the lapse of half an hour. Effects of occlusion experiments were observed also after denervation of the kidneys, destruction of the spinal cord below the lower cervical segment, splenectomy or adrenalectomy. In experiments with total ischemia of the liver or of an extremity, no corresponding pressor effect ensued after removal of the clamp, and variations in blood pressure in connection with occlusion of the splenic vessels are stated by the authors (without further explanation) to be due to changes in the volume of the spleen.

Contrary to COLLINS and HAMILTON, LEWIS, LEO and PRINZMETAL and their collaborators (1940—1941) did not find any correlation between the duration of the ischemia and the pressor effect; but, after extensive investigations, they confirmed the statement that occlusion of the renal vessels is often followed by a distinct pressor effect, which they had found in 83 out of 94 cases in experiments on cats. They found a similar pressor effect after the cess-

ation of total ischemia in dog and rat kidneys, but failed to find any corresponding pressor effect in experiments on rabbits. They therefore tried the transplantation of (1) normal and (2) ischemic rabbit kidneys on normal or nephrectomized rabbits, but with a similar negative result. They were thus forced to the conclusion that there must be a species difference between rabbits and the other test animals, manifesting itself in the inability of rabbit kidneys to form pressor substance after total ischemia. These results were confirmed by SCARFF and KEELER (1943), who likewise failed to find any rise of blood pressure in rabbits after the termination of temporary occlusion of the renal vessels.

For investigations into the nature of the pressor substance produced in total ischemia of the kidney, PRINZMETAL, LEWIS and LEO used perfusates obtained by repeated perfusions of ischemic kidneys with 1 ccm 0.9 % NaCl solution per gram of renal tissue. In corresponding perfusates of normal, non-occluded kidneys no pressor substance could be observed. In these experiments they found the following indications that the pressor substance is identical with renin: Both substances (1) are destroyed by boiling, (2) induce tachyphylaxis, (3) show identical pressor curves, (4) unlike adrenalin, give a pressor effect after the previous injection of 933 F (piperidomethyl-3-benzodioxane), (5) unlike tyramin, give a pressor effect after previous injection of cocain, and (6) yield a heatstable pressor substance on incubation with plasma.

DICKER, on the other hand, contended that the pressor substance produced after occlusion of the renal arteries of dogs for 24 hours could *not* be renin, in that it withstood lengthy boiling and was soluble in alcohol, which accorded with the view that it might be hypertensin (angiotonin). DICKER also found that it was stable even when kept for a considerable length of time as well as after oxidation with potassium permanganate, and that it was insoluble in ether.

The divergence between the findings of DICKER, on the one hand, and of PRINZMETAL, LEWIS and LEO, on the other, seems to be explained by the investigations of the latter authors. In experiments on cats, they extirpated both kidneys, one of which was quickly perfused with a warm Ringer's solution for the removal of the blood, whereupon both kidneys were incubated for 5 hours in the cat's abdomen. They found more renin in the blood-free than in the blood-containing kidney, which, on the other hand, contained a relatively large amount of heat-stable pressor sub-

stance (which however, they failed to find in perfusates of blood-containing, ischemic kidneys which had been clamped, but not excised). As a reason why more renin is found in the blood-free renal perfusates than in the blood-containing, they state — with reference to a coming publication — that the red blood cells contain a factor which tends to inhibit the formation of renin.

That in the preparation of perfusates of ischemic kidneys we obtain the substance which raises the blood pressure after the termination of a total ischemia is *a priori* probable. This supposition has in fact been corroborated by PRINZMETAL, LEWIS and LEO, who compared the pressor effects of perfusates of totally ischemic kidneys with the rises in blood pressure which they obtained with perfusates of corresponding kidneys released after the termination of the ischemia. As might have been expected, they found the least pressor effect with perfusates from those kidneys which were flooded with blood, with consequent discharge of pressor substance into the blood stream after removal of the clamps.

Author's Investigations.

Investigations into the effect of temporary total occlusion of the renal vessels.

In attempts to reproduce the above-mentioned occlusion experiments, cats narcotized with 0.05 g chloralose per kg of body weight were employed. As will be seen from Table 1, tests were made with the occlusion of (1) one or both arteries, (2) an artery and vein and (3) the entire renal pedicle. Such tests were made on altogether 11 cats, and varied in duration from 5 minutes to 7 hours. Only 3 of these animals reacted with a distinct rise of blood pressure (of 54, 12 and 46 mm, respectively) giving the characteristic curve seen after injection of renin (Fig. 1). In 7 out of the 11 cases no pressor effect was observed on the release of the clamp. This may be explained by supposing either that the cat was insensitive to renin — just as cat 5 was during the first occlusion test —, or that thrombosis had occurred in the renal vessels, so that the renal circulation was not restored on the release of the clamp. In some of the negative tests (cat 10, 30, 38) it was, however, found that neither of these suppositions held good, as these cats did react to renin, and, according to the criteria adopted by PRINZMETAL

Table 1.

Results of the temporary total occlusion of cat's renal vessels.

Nb.	Occlusion of	Duration of occlusion	Effect in mm Hg.
1	both arteries	5 minutes	0
»	» »	15 »	0
»	» »	15 »	0
»	» »	1 hour	0
2	one artery	15 minutes	0
»	» »	1 hour	4
»	» »	4 hours	6
3	one artery and vein	4 »	0
»	» » » »	15 minutes	3
4	one pedicle.....	15 »	0
»	» »	4 hours	54
5	» »	3 »	0
»	» »	2 »	12
6	» »	3 »	0
»	» »	2 »	0
7	» »	2 »	46
»	» »	2 »	0
9	» »	1 »	3
10	» »	$2^1/_2$ »	0
30	» »	$2^1/_4$ »	0
38	one artery and vein	7 »	6

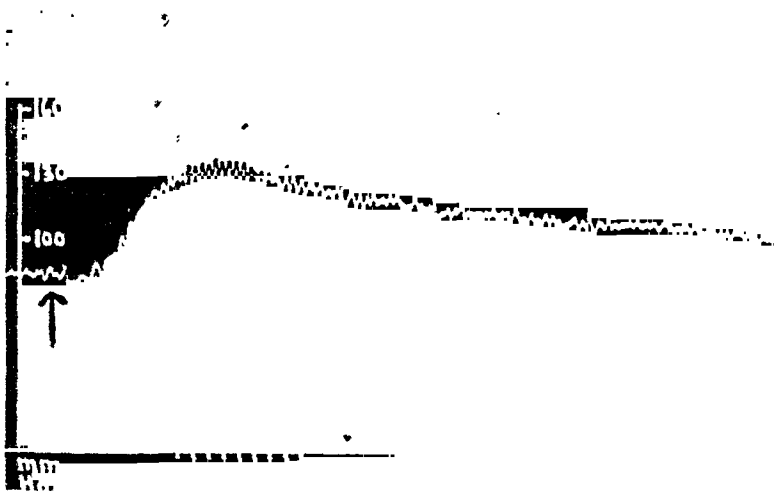

Fig. 1. Renin-like pressor curve after release of clamp of the one renal artery of a cat after 4 hours' clamping (Experiment 4). Time marking 30 sec.

et al. (bleeding from the kidney after a prick and rise of blood pressure after intrarenal adrenalin injection) the circulation had not been arrested.

Demonstration of renin in the perfusates from totally ischemic rabbit kidney.

As already indicated, previous investigators have failed to find any formation of pressor substance in the totally ischemic kidney of rabbits, and have therefore supposed that it reacted differently from dog, cat and rat kidneys to total ischemia. It seems, however, scarcely probable that there should be such a species difference, especially in view of the fact that hypertension can be induced in rabbits by partial renal ischemia. And, as it is well known that rabbits react more weakly to pressor substances than cats do, the author thought it desirable to investigate whether, by using cats as test animals, the formation of pressor substance could be shown also in ischemic rabbit kidneys.

The results of the intravenous injection into cats of 5 perfusates from rabbit kidneys occluded for 4—6 hours and of perfusates from the corresponding non-occluded kidneys are shown in Table 2.

Each of the perfusates was produced by perfusion of the kidney with as many cubic centimetres of Ringer's solution as it weighed in grams. To the Ringer's solution a little tricresol (3 drops to 500 cc) was added. The perfusion was repeated three

Table 2.

Effect of perfusates from occluded and non-occluded rabbit and cat kidneys on the blood pressure in cats.

Experiment	Kidneys from	Duration of occlusion	Dosage pr kg.	Effect in mm. Hg.
15	Rabbit 14	5½ hours	2.8 cc.	0
»	» »	0 »	» »	0
38	» 39	4 »	2.3 »	44
»	» »	0 »	» »	10
63	» 1	4 »	1.0 »	12
»	» »	0 »	» »	8
»	» 2	4 »	1.4 »	28
»	» »	0 »	1.8 »	14
»	» 3	4½ »	1.75 »	36
»	» »	0 »	» »	15
13	Cat 13	4½ hours	1.25 cc.	50
»	» »	0 »	» »	0
15	» »	4½ »	2.8 »	44
»	» »	0 »	» »	4
»	» »	6 »	» »	4
»	» »	0 »	» »	8

times with the same amount of fluid, whereupon the residual fluid in the kidney was carefully pressed out.

It will be seen that in 3 of the cases there is a *distinct liberation* of pressor substance from the totally ischemic rabbit kidney, the perfusate of which gives a much more marked effect than that from the control kidney. In the two remaining cases there is no difference; but in two similar tests on cats a distinct effect (which

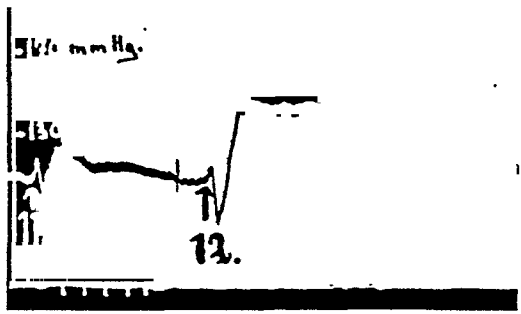

Fig. 2. Considerably greater effect after the injection on a cat of a perfusate from a rabbit kidney clamped for 4 hours (12) than of perfusates from the non-occluded fellow kidney (11) (Experiment 63).

was reproduced in tests with the same perfusates on a new animal) was obtained only in one of them, whereas both perfusates from the other cat were ineffective.

The difference between the effect of perfusates from occluded and non-occluded rabbit kidneys is shown by Fig. 2, where it will also be seen that the pressor effect is preceded by a transient depressor effect, which was observed also in the other cases.

The author also succeeded in showing a liberation of pressor substance in totally ischemic rabbit kidneys by incubation tests in vitro. In this case the kidneys were taken from urethanized rabbits or immediately *post mortem* from rabbits[1] killed by intravenous air-injection. After incubation in Ringer's solution (admixed with a little tricresol) for varying lengths of time at varying temperatures, a perfusate was then prepared in the manner above described. The perfusates were injected into cats narcotized with 5 cg chloralose per kg and pre-treated with an intravenous injection of ca. 0.1 mg ergotamin tartrate and ca. 1 mg atropin sulphate per kg of body weight.

[1] I am indebted to Professor WESTMAN, Karolinska Hospital, for kindly supplying me with rabbit kidneys.

Whilst in perfusates from kidneys perfused immediately after extraction no pressure substance could be shown, some pressor effect was observed in perfusates from kidneys incubated in a refrigerator at ca. 2° C (see Fig. 3). A stronger pressor effect was obtained with perfusates from the kidneys incubated at 37° C.

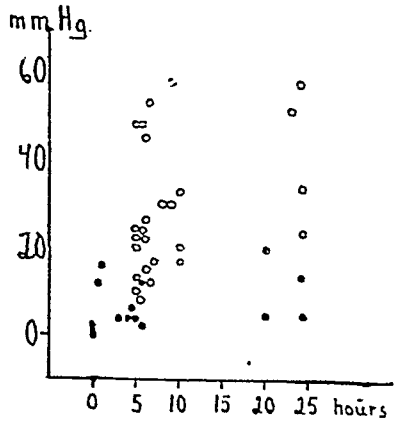

Fig. 3. Effect in mm Hg of the injection into cats of perfusates of rabbit — and in some cases cat — kidneys incubated in Ringers solution at ca. 2° (the black points) and at ca. 37° (the circles) for different lengths of time. (See the text.)

It is not possible, however, on the basis of Fig. 3, to form a reliable estimate of the relative effects of the perfusates incubated at 2° and at 37°, respectively, nor as to the part played by the duration of the incubation, seeing that the sensitiveness of the different animals as well as of the same animals at different times varies considerably. Moreover, the dosage, though usually 2 ccm per kg, was not always quite the same. But in tests where, with the aid of repeated injections of renin, it had been ascertained that the sensitiveness was fairly constant, and

Fig. 4. Renin-like effect of injection into a cat of a perfusate from rabbit kidneys incubated in tricresol Ringer (Experiment 29, Mark 11).

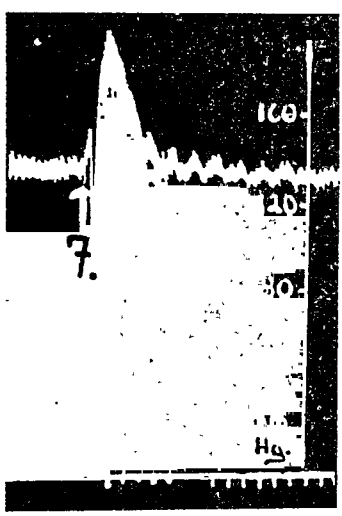

Fig. 5. Hypertensin-like effect of injection into a cat of a perfusate from rabbit kidneys incubated in tricresol Ringer. (Experiment 30, Mark 7.)

Fig. 6. Non-characteristic effect of injection into a cat of perfusates from rabbit kidneys incubated in tricresol Ringer. The pressure rises more steeply than in the reninin curve and in this respect resembles the hypertensin effect; but the rise of blood pressure is long maintained (Experiment 29, Mark 9).

where the dosage was strictly uniform, *a distinctly greater pressor effect was found after incubation at 37° than at 2°*, and also a *tendency to increase according to the duration of the incubation*. The configuration of the pressor curve showed some variation. It might be reninlike (Fig. 4) or hypertensin-like (Fig. 5) or intermediate between these two forms, as in fig. 6, where the rise is steep, whilst the fall is gradual. We must thus reckon with the possibility that the pressor substance found in the perfusates is not always of the same nature.

After thus showing the effect of perfusates from ischemic rabbit kidneys injected into cats, the author investigated whether a similar pressor effect could be obtained with rabbits as test animals. It was found, as expected, *that rabbits reacted much more faintly to the perfusates — and to*

Table 3.

Comparison between the effects of rabbit renal perfusates and of renin in cats and rabbits.

Experiment	Preparate	Effect in mm Hg on	
		Cat	Rabbit
28 og 29.....	Perfusate (4 hours 2°)	4 (2)	3 (2)
» » ».....	» (24 » 37°)	34 (1.7)	14 (2)
» » ».....	Renin	46 (0.17)	15 (0.24)
32 » 33.....	Perfusate (10 » 37°)	33 (2)	0 (2)
» » ».....	Renin	28 (0.15)	16 (0.15)
32 » 35.....	Perfusate (10 » 37°)	20 (2)	0 (2)
» » ».....	Renin	22 (0.15)	16 (0.15)
30 » 37.....	Perfusate (9 » 37°)	32 (2)	6 (2)
» » ».....	Renin	21 (0.15)	5 (0.15)
42 » 43.....	Perfusate (5 » 37°)	48 (2)	12 (2)
» » ».....	Renin	50 (0.15)	26 (0.15)

(The figures given in brackets after the BP values represent the dosage per kg of body weight in cc for the perfusates and in g dry substance for renin.)

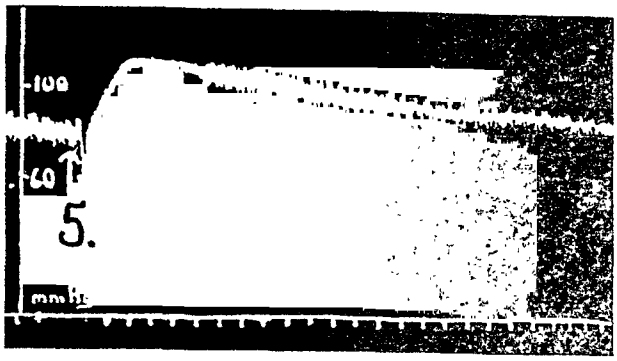

Fig. 7. Renin-like effect of the injection into a rabbit of a perfusate from rabbit kidneys incubated in tricresol Ringer. (Experiment 44, mark 5.)

renin — than cats. (Table 3.) In several cases, however, a distinct renin-like effect of the perfusates, such as that shown in Fig. 7, was observed.

Intensification of the liberation of renin after treatment of the kidneys with phenol, toluol, acetone or ether.

It having been found that, despite the admixture of tricresol, putrefaction set in when the kidneys had been left for some length of time in Ringer's solution at 37°, the author tried the admixture of *phenol.* It was then found that *the perfusate from kidneys thus incubated in "phenol Ringer" contained more pressor substance than the perfusates previously examined.* A direct comparison was then made between the pressor effect of kidneys (1) incubated in "tricresol-Ringer" and (2) incubated first for about half an hour to one hour in 2 % phenol and afterwards in "tricresol- Ringer"; the right kidney was used for the one method of incubation, and the left for the other, both kidneys being incubated at the same temperature (37°) and for the same length of time (usually about 5 hours) in each test. The results of these experiments are seen in Fig. 8, where 11 such comparisons are made. They show that a considerably greater pressor

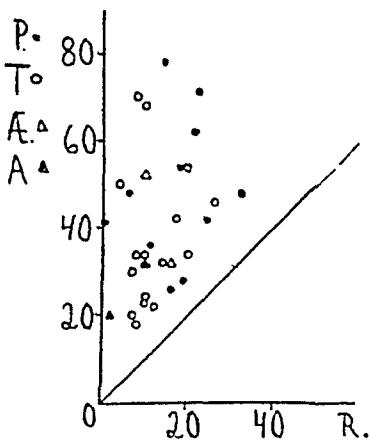

Fig. 8. Distinctly greater pressor effect of perfusates from rabbit kidneys pretreated with phenol (P), toluol (T), ether (AE) or acetone (A) and afterwards incubated in tricresol Ringer than of the same dosages of perfusates from the corresponding, not pretreated, but likewise incubated kidneys (R).

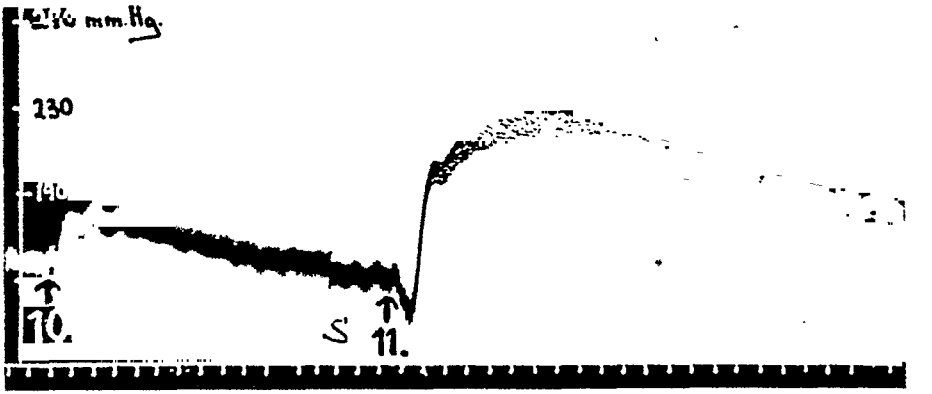

Fig. 9. The figure shows the difference in the effect of perfusates from a cat kidney pre-treated with phenol (11) and from the corresponding, not pre-treated, kidney (10) (Experiment 53).

effect is yielded by the perfusates from kidneys treated with *phenol*. The difference is shown also in Fig. 9, where it will be noticed that the curve has a typical renin form, apart from a primary depressor effect, which was frequently found, and which must presumably be attributed to the admixture of phenol.

Similarly as with the perfusates from the kidneys incubated in "tricresol-Ringer", *the effect of the perfusates from the kidneys treated with phenol tended* — up to a certain point — *to increase with the duration of the incubation*, giving, *e. g.*, a rise of 39 mm after 2 hours, 42 mm after 4 hours, 54 mm after 7 hours and 86 mm after 10 hours. However, when the incubation period was prolonged *e. g.* to 26 hours, a rise of only 64 mm was obtained.

That the effect of phenol is not specific is indicated by tests with similar *treatment of the kidneys with toluol, acetone or ether* (Fig. 10). They likewise showed a considerable increase of the pressor substance in the perfusates, relatively to the content thereof found in perfusates from kidneys treated with "tricresol-Ringer" (see Fig. 8).

In titration tests with the perfusates for the purpose of ascertaining the difference in the content of pressor substance in the differently treated kidneys, it was found that *the kidneys treated with phenol or toluol yielded 5—10 times as active a perfusate as those incubated in " tricresol-Ringer"*. After incubation for 5 hours, the activity of the perfusate was usually such that 0.25 cc (corresponding to 0.25 g of kidney) per kg had to be

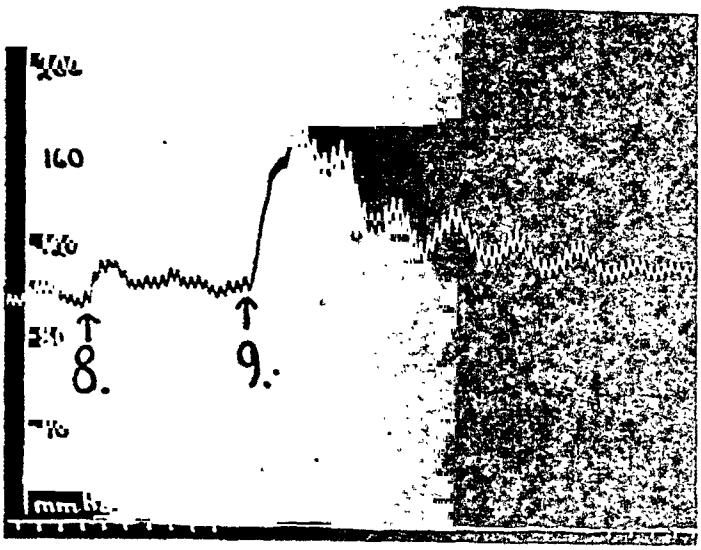

Fig. 10. The figure shows the difference in the effect of perfusates from a rabbit, kidney pre-treated with toluol (9) and from the corresponding, not pre-treated kidney (8) (Experiment 57).

injected into a cat in order to obtain a rise in blood pressure of 20—40 mm.

That the pressor substance is identical with renin is indicated by tests showing that the configuration of the curve is renin-like, that the substance is thermolabile and non-dialyzable and that, on the incubation of the substance with serum globulin[1], thermostabile hypertensin is obtained.

Finally, it should be mentioned that, on repeated injection, tachyphylaxis phenomena appear. Such phenomena, however, set in more slowly with perfusates than with extracts from kidneys, which is presumably due to the greater purity of the perfusates.

Summary.

1. A review is given of previous investigations into the liberation of renin from totally ischemic kidneys, and it is confirmed that a pressor effect can be obtained on the termination of the temporary total occlusion of the renal vessels in cats.

2. It is shown that a liberation of pressor substance after total occlusion of the renal vessels can be obtained also in *rabbits*, and that pressor substance is found also in perfusates of cat and rabbit

[1] I am indebted to Dr Edman for kindly supplying me with globulin.

kidneys incubated in Ringer's solution. In perfusates of rabbit kidneys immediately after extirpation, no, or scarcely any, pressor substance is observed; but after incubation at ca. 2° C. some pressor substance is found, and after incubation at 37° considerably more, with a tendency to increase in amount according to the duration of the incubation.

3. It is shown that the content of pressor substance in perfusates of incubated rabbit kidneys is considerably increased (is approximately 5—10 doubled) after pre-treatment of the kidneys with phenol, toluol, ether or acetone. The pressor substance in these perfusates must be considered to be identical with renin.

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On the Anticoagulant Activity of Heparin and Synthetic Polysaccharide Sulfuric Acids.

By

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Received 30 August 1944.

Different methods exist for measuring the anticoagulant activity of heparin, but so far the only one yielding a *curve* from which the strength may be calculated, is the method described by FISCHER and SCHMITZ (1932) who used chicken plasma and chicken thrombokinase (as a rule a dilute embryonic extract) cf. FISCHER and ASTRUP (1938). This method was at first used without a comparison sample, but later it was found, that the results differed when made on different plasmas, and a standard for measuring heparin activity was therefore established (ASTRUP and BEHRNTS JENSEN 1938). Many laboratories will find the use of chicken plasma inconvenient, but no method using more accessible plasmas has been as accurate as this one.

In investigations on the effect of different amounts of thrombokinase on the clotting time of recalcified oxalated ox plasma containing heparin or synthetic polysaccharide sulfuric acids (ASTRUP, GALSMAR and VOLKERT 1944) it was found that the resulting curves deviated from the straight lines at the lower concentrations of thrombokinase. Thus it was evident, that in order to obtain reliable measurements of anticoagulant activity, it was necessary that a large amount of thrombokinase was present, since in the presence of smaller amounts the clotting system responded too vigorously to the addition of an anticoagulant. This was especially true in the case of heparin, while the synthetic anticoagulants showed only minor deviations from the straight

lines. Theoretical reasons too make it necessary to assume, that the reactions during the clotting proceed more uniformly in the presence of a surplus of thrombokinase (i. e., an amount sufficient to yield the so-called "prothrombin-time"), and that the treatment of the results obtained in this manner would be the simplest. Well defined and reproducible conditions are obtained only under such circumstances and it must be expected that small amounts of the inhibitory substances show a more uniform action in the presence of a surplus of thrombokinase since, for example, probable dissociation processes may be ignored.

On the basis of such considerations, and with our previous experiments in mind, we have tried to find a method based on ox plasma, which also would lead to a curve, and thus yield an increased accuracy over previous methods using such plasma. This has been attained by using thrombokinase prepared from ox brain, cf. ASTRUP (1944).

Experimental.

The oxalated ox plasma used (prepared from blood containing 15 ml of 20 per cent potassium oxalate per liter) must be fresh and stored at 0° (not over three days) and filtered immediately before use. The optimal amount of 1.5 per cent $CaCl_2$ sicc. is used for recalcification, as a rule 0.20—0.30 ml per ml of plasma. A transparent water bath (37°) is used.

The thrombokinase is prepared from a fresh and cleaned ox brain, which is passed once through a meat chopper. The mass is pressed through two layers of gauze, and to 100 g of the resulting pulp 300 ml of physiol. NaCl solution and 1 ml of 90 per cent phenol are added. The mixture is treated 24—48 hours in a ball mill, yielding a fine suspension which may be stored several months at 0°.

The optimal amount of calcium chloride solution and 0.1 ml of the thrombokinase are placed in our usual clotting tubes (80×15 mm). One ml of a plasma mixture (0°) (composed of 4.5 ml of plasma and 0.5 ml of a solution of the anticoagulant in physiol. NaCl) are added rapidly from a pipette, and the clotting time determined in seconds. The tube is gently moved during the clotting. The clotting time chosen is the time when the fluid becomes clear with the formation of agglutinated particles. This point can be more sharply observed than the point of coagulation of the whole mixture, and may also be assumed to represent a better defined state of the clotting process, signifying the time when sufficient fibrin has been formed for agglutination of the suspended particles of calcium oxalate. These two points coincide only in the case of the shortest clotting times.

The concentration of the solutions of anticoagulant used is varied n order to obtain a sufficiently accurate curve (in the case of heparin

(about 50 per cent pure) solutions of 25, 50, 75 and 100 mg per cent are used). The approximate strength of the anticoagulants may be determined by the method described by ASTRUP, GALSMAR and VOLKERT (1944) before the final solutions are made.

From the mean values of the clotting times (t) obtained, and the concentration (c) of the anticoagulant, a curve is drawn with c as abscissae and $\log t$ as ordinates. Only clotting times under 1—2 minutes give sufficiently straight lines. All measurements must be checked against a sample kept as a standard.

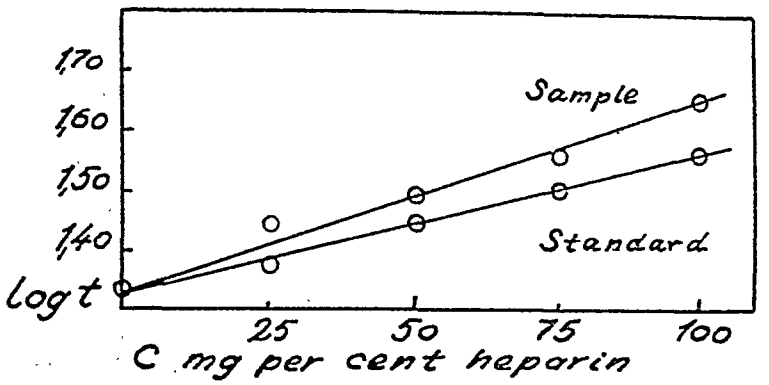

Fig. 1. Assay of heparin on ox plasma containing a surplus of thrombokinase.

Fig. 1 shows the curves, and it is seen that the points lie on straigth lines. From the slope of these lines the potency of the unknown sample is calculated as $\frac{3.8 \times (1.65 - 1.33)}{(1.56 - 1.33)} = 5.3$. With the chicken plasma method a value of 4.8 was found. Several other samples measured with the chicken plasma method were tried, and the values determined were always found to correspond to the original values within 15 per cent. Thus the new method seems to be comparable to that of the chicken plasma method and may be used instead. It may be found more convenient in most laboratories, but an accurate determination of the clotting times is easier with the chicken plasma method.

Corresponding experiments were made with the synthetic polysaccharide sulfuric acids of cellulose, starch and chitin. They yielded also straight lines, see Fig. 2. In all cases the amount of anticoagulant necessary is lower than for heparin, and this is especially true of the cellulose derivative. This is in accordance with the previous results showing that large amounts of thrombokinase have a far greater effect on heparin than on the synthetic anticoagulants. It is therefore impossible to express by a figure

the relation between the potencies of such substances, and to use heparin as a standard for the assay of synthetic anticoagulants or vice versa. While the synthetic substances are found to be more

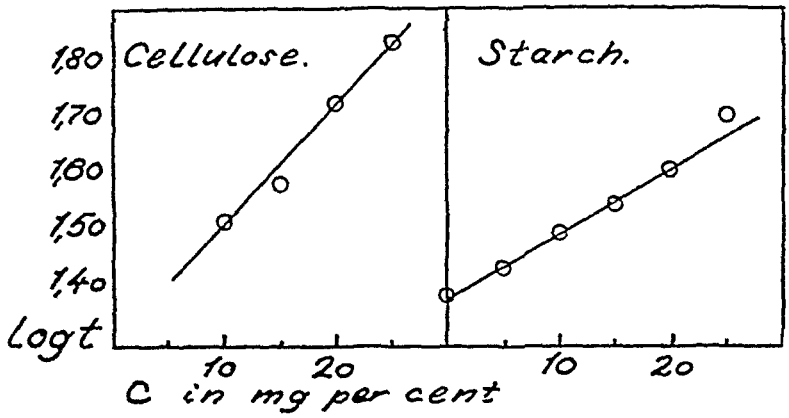

Fig. 2. Effect of cellulose and starch sulfuric acids on ox plasma in the presence of a surplus of thrombokinase.

potent than heparin when large amounts of thrombokinase are used (as in this method of measurement) the reverse will be found to be true in the presence of small amounts.

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Summary.

A method convenient for the determination of the anticoagulant strength of heparin and synthetic polysaccharide sulfuric acids is described. Ox plasma and a large excess of thrombokinase are used, and the accuracy of the method seems comparable to that of the chicken plasma method. Under such conditions the synthetic substances are more powerful anticoagulants than is heparin.

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Reticulocyte Ripening Substances in Pregnant Rats and their Fetuses.

By

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In a series of investigations by RUTH PLUM, not yet published it has been shown that rats exhibit a gradual rise in the reticulocyte ripening substances of the plasma during pregnancy.

The question then arises whether this rise in the reticulocyte-ripening substances is due to an increased production in the mother organism, or whether it is caused by an even larger production of ripening substances in the fetuses. Both suppositions are conceivable, the reticulocyte ripening substances being extremely diffusible, so that a passage through the placenta from the maternal to the fetal circulation or the reverse might very well take place.

Judging by previous investigations on the amount of ripening substances in animals with an increased erythropoiesis (C. M. PLUM, 1943 b) in which an increase in the ripening substances occurred, the most reasonable presumption is that the increased amount of ripening substances in the maternal blood is due to a diffusion from the fetal to the maternal circulation, since there is a greatly increased erythropoiesis in fetal life.

Unfortunately, it proved very difficult to procure fetal blood from rats for plasma investigations on the quantity of ripening substances, with the technique hitherto adopted (C. M. PLUM, 1942 a, b) for the determination of the amount of ripening substances in the plasma. Another procedure was therefore chosen. On the basis of previous investigations on the content of ripening substances in various organs (C. M. PLUM, 1944 a, b), the amounts

of ripening substances in the maternal and fetal livers as well as in the placenta were compared. The placenta was included because the exchange of the diffusible substances of the two organisms takes place here.

White female rats with an average weight of 250 g were used for the experiments.

The technique adopted for investigations on reticulocyteripening substances in plasma and in organ extracts has been described in more detail by C. M. PLUM (1942, 1944).

Through daily examination of the vaginal smears for some time the cycle of the rats was followed. When it had passed through a couple of periods, a male rat was let in to the female rat corresponding to the oestrus. If spermatozoa were found in the vaginal smears and a succeeding cessation of the cycle occurred, it was assumed that conception had taken place. On the 20th day after conception the animals were put under deep anesthesia and strapped down on their backs. The abdominal wall was cut open and with a record syringe mounted with a cannula about 8 ml of blood were drawn from the vena cava inf. Then the uterine horns were cut open and the fetuses removed. The funiculus umb. was cut off close to the placenta. Next the living fetuses were cut open and the livers removed. Finally the liver was removed from the mother animal. All the organs were now minced finely and put in an exsiccator (max. 42° C.) for 24 hours.

After this the organ extracts were made as previously described (C. M. PLUM 1944 a and b).

The determinations on the plasma were made immediately after the killing of the animals.

The results of the experiments will appear from the table. As will be seen, an increase of 15—20 % is found in the amount of ripening substances in the plasma of the pregnant rats. The increased production is found in the last fetal days, in which a very

Normal female rats				Mother				Fetus				
Plasma		Liver		Plasma		Liver		Placenta		Liver		Number
0.82	0.85	0.27	0.89	0.96	0.98	0.30	0.90	0.35	1.00	0.40	1.17	7
0.81	0.83	0.26	0.87	0.98	1.00	0.32	0.91	0.35	0.99	0.39	1.12	6
0.78	0.80	0.25	0.85	0.96	0.99	0.30	0.92	0.36	1.04	0.41	1.16	8
0.86	0.88	0.28	0.90	0.94	0.96	0.31	0.90	0.37	1.06	0.41	1.21	7
0.83	0.85	0.27	0.87	0.98	1.00	0.32	0.92	0.37	1.07	0.42	1.23	8
Average 0.82	0.84	0.26	0.88	0.96	0.99	0.31	0.91	0.36	1.03	0.41	1.18	

Results of investigations on the content of ripening substances expressed by the ripening index in the plasma of normal and pregnant rats and in their livers and placentae. The second column shows the activated ripening index, i. e. the ripening index after the addition of tyrosine (C. M. PLUM, 1944 a).

lively erythropoiesis was observed at the same time (JACOBSEN and PLUM 1942 a). The increase in ripening substances, however, takes place gradually in the plasma of the mother organism during pregnancy (RUTH PLUM).

While in normal individuals within the same species of animal it is seen that a high reticulocyte number corresponds to a low concentration of ripening substance in the plasma (the ripening index) and the reverse (C. M. PLUM, 1943 a and 1944 a), no reduction of the amount of reticulocytes is seen in the pregnant rats, though it might have been anticipated owing to the increase in ripening substances. In the fetuses the large amount of reticulocytes is found in the last fetal days (SEYFARTH and JÜRGENS, 1928), JACOBSEN and PLUM, 1942 a), and here a high ripening index was to be expected, for here there must be said to be an increased erythropoiesis (PLUM, 1943 b).

An examinaton of the extracts of the dried organs (1 g organ — 10 ml of 0.9 % NaCl solution), as was to be expected, showed an increase in the mother liver, corresponding in order of magnitude to the plasma content of ripening substances. The investigations exhibited an increase in the finished ripening substances (C. M. PLUM, 1944 a, b) in the liver while the activation with tyrosine is comparatively less in pregnant than in non-pregnant rats.

The amount of ripening substances in the fetal livers is considerably larger than in the liver of the mother organism. Curiously enough, it is found that the amount of ripening substances in the placenta is of an order of magnitude that lies halfway between that found in the mother organism and that of the fetal livers.

Thus there seems to be a close interaction between the mother organism and the fetuses, since it must be supposed that the fetuses themselves are able to produce the ripening substances in their preformed shape. If the preformed ripening substances are to be utilised by the organism, they must be coupled with tyrosine or a tyrosine-like substance (JACOBSEN and PLUM, 1943), so the mother organism must produce or in other ways see to it that the fetuses receive the requisite amount of activation substances. These substances must either be diffused through the placenta to the fetuses, or the preformed ripening substances must be diffused from the fetus to the mother. The latter assumption is, however, little likely, since no increased amount of preformed ripening substances is found in the maternal blood.

On the basis of the experiments here discussed in which a deter-

mination was made of ripening substances in the livers of fetuses and of normal rats, there is every reason to believe that the blood of the fetuses actually does contain an increased amount of ripening substances, as it has previously been shown (PLUM, 1944 a and b) that there is a close correlation between the amount of ripening substances in the organs and in the plasma.

Summary.

Through a series of investigations on the amount of reticulocyte ripening substances in the livers of pregnant rats and their fetuses as well as in the placentae, it is shown that an increased formation of ripening substances takes place in the fetuses. This increase manifests itself by an increased amount of ripening substances in the maternal blood and liver as well as in the fetal liver.

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The Purification of Aminopolypeptidase from the Cattle Muscle.

By

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In the purification of aminopolypeptidase from the pyloric mucosa of hog (ÅGREN, 1942) it was soon found that one of the main difficulties was to separate the peptidase activity from the mucin present in all pyloric extracts. As a preliminary study to the further purification of the enzyme it was decided to study the properties of a closely allied enzyme, the aminopolypeptidase of cattle muscle, a more mucin-free material. It had previously been demonstrated that the peptidase activity of the muscle was rather high (ÅGREN, 1940), and the enzyme was accordingly prepared from this material.

Experimental procedures,

The enzyme activity was determined according to LINDERSTRÖM-LANG and HOLTER (1931, 1932) using a 0.2 M solution of alanyl-glycine of pH 7.4 as substrate. One unit of activity was defined as the amount which, after 30 minutes of digestion at pH 7.4 and 37° C, gave an increase in amino nitrogen corresponding to 1 c. mm. of n/20 HCl in 90 per cent alcohol. Protein nitrogen was determined by the micro-Kjeldahl procedure. Traces of ammonium sulphate or nitrogen bases contaminating the solutions in some steps of the purification necessitated a preliminary precipitation of the protein with trichloracetic acid. Usually 1 ml of solution containing $>$ 0.2 mg of nitrogen was precipitated with 2 ml of 10 % trichloracetic acid. After heating for two minutes at 100° C. the mixture was cooled in tap water, the precipitate was centrifugated, washed twice with 2 % trichloracetic acid and hydrolyzed with concentrated sulphuric acid.

Results.

Method of extraction. Diaphragm muscle from cattle was chilled in the slaughter-house immediately after the death of the animals, and cut very fine in a mincer. The minced muscles were suspended in 4 parts of water and shaken for 30 minutes at 37° C, chilled to room temperature, and successively filtered through gauze and a thin layer of Standard Super Cel on a 30 cm Buchner funnel. In this way a clear, red extract was obtained, which contained 1.7 units per c. mm. of extract. Thus, the peptidase activity of cattle muscle is about half as high as that of the pyloric mucosa of the hog, when the digestion of alanyl-glycyl-glycine is taken as reference. Preliminary trials to a further purification by precipitation with magnesium sulphate or ammonium sulphate at different pH were less successful. In the range of pH 4.0—5.0 most of the enzyme activity was lost. At pH 6—8 $MgSO_4$ did not precipitate the extract, and it was not possible to separate the enzyme from the muscle hemoglobin with ammonium sulphate. A further purification without considerable loss of activity was obtained by precipitation with lead acetate. A rather heavy, white precipitate was obtained. The optimal conditions usually corresponded to precipitation with 0.5 N lead acetate, $^1/_{30}$ of the volume of the muscle extract. After centrifugation and neutralization to pH 7.4 the centrifugate was dialyzed against water for 24 hours at 0° C. The solution in the dialysing tube contained 0.20 mg nitrogen and 1,400 units of activity per ml. By this procedure the enzyme was purified about 7 times.

Since different experiments toward a further purification had not led to any separation of the muscle hemoglobin and the enzyme, it was decided to try cataphoresis at different pH for the same purpose. As a preliminary step to such experiments it was necessary to investigate the stability of the enzyme at different pH and temperatures. In the experiments a lead-precipitated muscle extract was used. In Table 1 the pH-stability of the enzyme at room temperature (20° C.) is given. After the

Table 1.

The pH stability of aminopolypeptidase stored at room temperature (20° C.) for different times and at different pH.

Time in minutes	Activity in units per mm³ solution at pH									
	4.32	4.40	4.63	4.82	4.90	5.07	7.09	8.18	8.60	9.42
30	0.70	0.74	1.2	1.5	1.5	1.5	—	—	—	—
120	—	—	—	0.85	—	1.5	—	—	—	1.5
240	—	—	—	—	1.4	1.5	1.4	1.5	—	1.4
1,200	—	—	—	—	—	—	—	1.5	1.5	1.3

incubation time the solutions were neutralized to pH 7.4 and used for the digestion tests.

At room temperature and between pH 4.9 and 9.4 the enzyme is stable for about 84 hours. At pH 4.8 and 0° C. the activity was constant for at least 24 hours. The stability of a neutral enzyme solution kept for 15 minutes at higher temperatures is demonstrated in Table 2.

The first series of cataphoretic experiments was carried out in the Tiselius analysis apparatus. 100 g of muscle in experiments

Table 2.

The temperature stability of a neutral solution of aminopolypeptidase heated to higher temperatures before digestion.

Time in minutes	Activity in units per mm³ solution		
	50°	60°	70°
15	1.5	0.4	0

1—4, or 200 g in experiments 5—7, were extracted and precipitated with lead acetate as described above. After vacuum concentration at 15° C. to 60 ml the solution was dialyzed at 0° C. for 24 hours against water and further concentrated in a vacuum to 8 ml. For each experiment 2 ml of a similar solution were diluted to 15 ml with the necessary buffer. All the experiments were run for 6 hours with approximately the same ionic strength of buffers, and the same strength of current. In Table 3 some of the experimental data of the cataphoresis are given. In Table 4 the enzymatic activities of the different cell contents at the end of the experiment are demonstrated.

From the figures in Tables 3 and 4 it can be seen that cataphoresis enables a separation of aminopolypeptidase from the muscle haemoglobin and a yellow protein to be carried out on a small scale. A comparison of the figures also shows that the optimal conditions for a separation would be obtained by cataphorizing at about pH 6, where the anodic migration of the enzyme as demonstrated by the enzyme values of Table 4 was still maximal and the cathodic migration of muscle haemoglobin and the yellow component were also rather rapid. After a six hours run the + 2 and + 1 cells of experiments 4—7 did not contain any coloured material. No attempt was made to deter-

Table 3.

Experimental data and qualitative results of cataphoretic experiments.

Experiment No.	pH	Migration of			buffer
		enzyme	muscle haemoglobin	"yellow protein"	
1.....	7.02	anodic	anodic	andodic	phosphate
2.....	6.70	»	cathodic	»	»
3.....	6.44	»	»	cathodic	»
4.....	6.10	»	»	»	»
5.....	5.84	»	»	»	»
6.....	5.26	»	»	»	acetate
7.....	5.00	»	»	»	»

Table 4.

Enzymatical analysis of the cataphoretic experiments in the analytical apparatus.

The anode cells are termed + 1 and + 2 counted from the bottom cell, 0, and, in the same way the cathode cells are termed — 1 and — 2.

Experiment No. (same as in Table 3.)	Activity in units per mm³ solution in cell No.				
	— 2	— 1	0	+ 1	+ 2
1.....	0	0	31	32	23
2.....	0	0	36	26	38
3.....	0	0	31	24	29
4.....	0	0	32	27	36
5.....	0	5.7	74	54	69
6.....	0	43	83	71	34
7.....	0	50	80	70	19

mine the migration velocity of the enzyme quantitatively since the fraction moving in the anodic direction between pH 6.4 and 5.0 consisted of at least two substances migrating with about the same velocities. From the enzyme values of the + 2 cell contents experiments 5—7 Table 4, it can roughly be estimated that the isoelectric point of the enzyme would be close to 4.6. The purification obtained from the cataphoretic separation was rather good. If the activity per mg of nitrogen was calculated, there was a sixfold increase and the yield was 50 per cent, if the enzyme activity of the two anode cells were taken together. The figures were calculated from experiment 5, where the total nitrogen of cells + 2 and + 1 were respectively 2.0 and 3.8 mg per ml solution. A comparison of the enzyme values of Table 4 seems to indicate that some cathodic migrating protein or proteins were to a certain degree acting as a carrier of aminopolypeptidase

in the same manner as, in similar conditions protamin acts as a carrier for carboxypolypeptidase (ÅGREN and HAMMARSTEN 1937).

To obtain material in amounts required for further purification after the cataphoresis, it was necessary to carry out the separation of enzyme and coloured material in the Tiselius preparative apparatus. For each experiment 1,000 g muscle were extracted with 4,000 ml of water and purified with lead acetate as above, and after concentration to 150 ml, dialysis and filtration, concentrated to a final volume of 40 ml. Finally, the solution was diluted to about 100 ml with the necessary phosphate buffer so that the bottom cell, filled with glass beads, and four of the eight cells of the apparatus, could be filled up. All the ten experiments were carried out at pH 5.8 and run for 48 hours. By compensation in the usual way it was possible to obtain the anodic part of the apparatus practically free from coloured material. The enzyme activity filled 3 of the 4 anode cells of the apparatus. At the end of each experiment the content of the anode cells was concentrated in vacuum to half the volume, dialyzed against water for 24 hours, and again concentrated to about half the volume and stored at — 20° C. Several attempts to prepare a dry, stable powder by precipitating a cold enzyme solution with cold acetone failed. About 70 % of the activity was lost. Apparently, in spite of all precautions, the preparations were denaturated. At — 15° C the concentrated, cataphorized solutions could be stored for more than a year without any loss of activity. Further attempts to purify the cataphorized, concentrated material by lead acetate did not meet with success. Some impurities could be removed by heating the solutions at 50° C. for 3 minutes. Without any loss of activity more than 20 % of protein impurities were precipitated. A further progress was obtained by fractionated precipitation with ammonium sulphate. A very essential point in this procedure was to maintain a slightly alkaline reaction, pH about 8, which was obtained by adding $NaCHO_3$ in substance to a concentration of m/10. Solid ammonium sulphate was added to 0.6 saturation, whereupon a fine precipitate was formed. After 15 minutes the precipitate was removed by centrifugation at 15,000 r. p. m. To the centrifugated clear solution solid ammonium sulphate was added to 0.7 saturation. The two precipitates were separately solved and dialyzed at 0° C. for 4 hours against m/200 Na_2HPO_4. The

Table 5.

Fractionated ammonium sulphate precipitation of the concentrated solution from cataphoresis No. 10 in the preparative apparatus.

Sample	Volume in ml	Activity in units per mm² solution.	Protein nitrogen in mg per ml
Cataphorized solution ..	25	Diluted 1:100=0.71	3.5
0.6 saturation precipitate solved in	10	» 1:200 =0.85	2.5
0.7 saturation precipitate solved in	22	» 1:100=0.14	2.2

contents of the dialyzed tubes were analyzed. The results of a typical fractionation are given in Table 5.

In a cataphoretical examination of the fraction precipitated at 0.6 saturation with ammonium sulphate there seemed to be only one component present in the solution. Total carbohydrates estimated by the method of TILLMAN-PHILIPPI in the modification of SÖRENSEN and HAUGAARD was present to less than 1 %. The usual colour reactions for amino acids were all positive. Quantitative data from the different steps of the preparation are given in Table 6.

Discussion. Through the method outlined above the aminopolypeptidase of cattle muscle has been purified about 70 times and obtained in a fairly pure state. The yield of the method was 25 %. The greatest loss of activity occurred in the cataphoresis, possibly through the property of some cathodic migrating protein to act as a carrier for the aminopolypeptidase. In this connection may also be mentioned the lack of parallelism between the yields and purity of the material purified by the analytical and preparative apparatus of Tiselius. The analytical apparatus gave better results both qualitatively and quantitatively. The possible presence and nature of an active group in the enzyme will be further investigated in connection with studies into the properties of the aminopolypeptidase of the hog's pyloric mucosa. In several respects the two enzymes seem to be closely related. Highly purified solutions of aminopolypeptidase from the cattle muscle and the hog's pyloric mucosa both migrate anodic to an acid reaction whereupon incipient inactivation makes further experiments impossible (pH about 4.6). Several attempts to reactivate the inactivated solutions failed. Both enzymes are precipitated at the same concentrations of lead acetate and ammonium sul-

Table 6.

The purification of aminopolypeptidase.

Procedure	No.	Activity in units per mm^3 solution	Mg protein nitrogen per ml	Activity in units $\times 10^3$ per mg nitrogen	Yields in units $\times 10^5$
1,000 g of frozen muscle extracted with 4,000 ml water, filtered through gauze and clearified with Standard Super Cel. Volume 4,000 ml	1	1.70	1.5	1.05	68
To No. 1 was added $^1/_{30}$ by volume of 0.5 N lead acetate, the precipitate immediately centrifuged, after centrifugation neutralized to pH 7. Volume 4,100 ml	2	1.4	0.20	7.0	57
No. 2 was concentrated to 150 ml in vacuum at 15°, dialyzed for 24 hours against water, concentrated to 40 ml and cataphorized in the preparative apparatus for 48 hours at pH 5.8. The content of the four anodic cells concentrated to 25 ml ...	3	71	4.5	16	17
No. 3 was kept at 50° for 3 minutes. The precipitate centrifuged and washed with water. Volume 25 ml	4	71	3.5	20	17
No. 4 was brought to 0.60 saturation of ammonium sulphate in m/10 $NaHCO_3$ by adding the solid salts. The precipitate solved in water and dialyzed against m/200 Na_2HPO_4 at 0° for 8 hours. Volume 10 ml ...	5	170	2.5	68	16

phate and in both cases a high degree of purification is obtained. The enzyme from cattle muscle is a carbohydrate-free protein. This makes it probable that the enzyme from the hog's pyloric mucosa, which is presently investigated, also is a protein with a low carbohydrate content. This is of some interest since the one of the main problem in the purification of the enzyme from the pyloric mucosa has been to settle the question wether the fairly high content of mucoides present during all steps of the purification is an integrating part of the enzyme.

Summary.

The aminopolypeptidase from the cattle diaphragm muscle has been obtained in a state of high purity. The enzyme is a carbohydrate-free protein with an isolelectric point close to pH 4.6.

The writer is indebted to the Johan and Therese Anderssons Minne foundation for grants which supported the present investigation. He further acknowledges the valuable assistance of Mrs Ågren throughout the investigation.

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A Note on the Action of Lobeline, Nicotine and Acetylcholine on the Afferent Nerves of the Tongue.

By

YNGVE ZOTTERMAN.

Received 28 September 1944.

HEYMANS, BOUCKAERT and DAUTREBANDE (1931) showed that the well-known effect of lobeline and nicotine upon respiration is due to the action of these substances in minute quantities on the chemoreceptors of the carotid sinus body. In 1939 EULER, LILJESTRAND and ZOTTERMAN studying the action potentials led off from Hering's nerve presented evidence for the view that these alcaloids exert their stimulating action on the chemoreceptive mechanism centrally to the point, where oxygen lack and carbon dioxide stimulate. This view was further strengthened when the same authors (1941) were able to show that 5—10 μg acetylcholine introduced into the carotid sinus of the cat via the external carotide artery gave rise to a massive volley of short duration of chemical impulses.

The seat of action is best given by fig. 1. which shows two alternative schemes:

A. The afferent neuron of Hering's nerve terminates around a ganglion cell located inside the carotide body. The adequate stimuli act upon the chemoreceptor which is situated in intimate contact with the arterial blood-flow, while lobeline and nicotine etc. act directly upon the synapse.

B. The afferent neuron terminates around the specific chemosensitive cells forming a junction which is sensitive to lobeline etc.

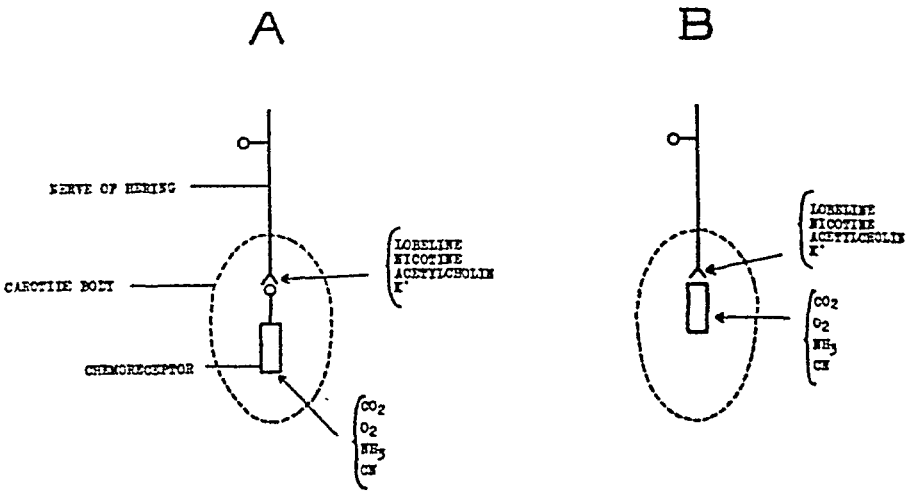

Fig. 1.

In both cases the alcaloids are believed to exert their action directly upon the peripheral junctions of the afferent neurons from which we obtained the action potentials which we recorded. The very high frequency of response far exceeding the highest frequency observed when using very strong adequate stimuli, also speaks in favour of this view.

These findings raise the question whether the action of these alcaloids on the chemoreceptive mechanism of the carotide body is of a specific nature or whether they exert a similar action upon other peripheral afferent mechanism.

An easy way of approach seemed to be to study their effect upon the various receptor mechanisms of the tongue of the cat. Specific action potentials due to thermal, chemical and tactile stimuli can be recorded from the lingual and glossopharyngeal nerve of the cat (ZOTTERMAN 1935, 1936). Small branches of these nerves were dissected out and freed from connective tissue and cold water, and diluted acetic acid was applied to the tongue in order to ascertain that the respective afferent fibres were functioning normally. Injections of lobeline, nicotine and acetylcholine were now introduced into the lingual artery via a cannula inserted into the thyroid artery. No effect whatever could be seen when applying in this way lobeline and nicotine in doses from 5 μg—0.5 mg. Neither the taste and cold fibres nor the tactile fibres showed any trace of an augmented activity.

With acetylcholin the effect was very much the same, the

only difference being, that this alcaloid produced fibrillations in the tongue which secondarily elicited a certain amount of tactile impulses. The activity of these tactile fibres was however of a low degree corresponding to the mechanical effect elicited by the acetylcholine.

Thus neither lobeline, nicotine nor acetylcholine when introduced intraarterially produce any stimulating affect upon any afferent mechanism of the tongue. The action of these alcaloids upon the chemoreceptive mechanism of the carotide sinus body must therefore be considered to be of a quite specific kind.

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Piperidine as a Normal Pressor Constituent of Human Urine.

By

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Received 6 November 1944.

ABELOUS and BARDIER showed in 1908 that ether extracts of normal urine contain a pressor principle which they named urohypertensin. Certain observations in connection with a study on the pressor activity of normal and hypertensive urine (EULER and SJÖSTRAND, 1944) indicated that the chief pressor activity of urine cannot be ascribed to isoamylamine, a pressor amine isolated from urine by BAIN (1914). An investigation of the nature of the ether soluble pressor substance in cow's urine led to the isolation in considerable amounts of piperidine as crystallized picrate, which could be identified chemically and by biological tests (EULER, 1944).

In the present paper it is shown that piperidine also accounts for the major part of the nicotine-like pressor activity in human non-smokers' urine.

Methods.

The urine was collected for 24 hour periods from normal healthy persons. Since it has been observed by HELMER, KOHLSTAEDT and PAGE (1939) that smokers' urine regularly contains nicotine, which has an action qualitatively identical with that of piperidine, it has become necessary to use only non-smokers urine for these experiments. The urine was slightly acidified with sulfuric acid and concentrated in vacuo to about one fifth of its volume. It was then precipitated with three volumes of ethyl alcohol and left over night in the refrigerator. The alcohol was evaporated and to the watery solution sodium hydroxide was added until pH 10—11. The extract was then subjected to

fluid extraction with ether for some three hours and the ether shaken with 5—10 ml of water to which sulfuric acid was added until the reaction remained slightly acid.

After removal of the ether the extract, of which 1 ml corresponded to 100—200 ml of urine, was tested biologically against piperidine hydrochloride as a standard on the blood pressure of the cat in chloralose anaesthesia. The specificity of the pressor action was tested by repeating the testing after treatment of the animal with cocaine hydrochloride in a dose of 10 mg pr kg intramuscularly, which abolishes the action of certain pressor amines such as tyramine and isoamylamine (TAINTER, 1933). In some cases a moderate reduction in activity was observed, but mostly the pressor action was unchanged.

The extracts were also examined as to their biological action on the isolated rabbit's intestine. On this test object isoamylamine has very little action even in high doses, whereas piperidine — like nicotine — produces intense stimulation.

Results.

a) *Isolation and identification of the pressor principle.*

The pressor activity of the urine extracts was found to be distillable with steam at alkaline reaction (pH 10). Extracts obtained by fluid extraction with ether were accordingly concentrated in vacuo to a small volume and distilled after addition of sodium carbonate to pH about 10. The distillate was neutralized with picric acid, and alcohol added to about 50 %. The solution was then left to crystallize at low temperature. The different fractions of crystalline picrates obtained were biologically tested and the most active fraction further purified. After repeated recrystallizations uniform needle-shaped crystals were obtained, undistinguishable from piperidine picrate prepared in the same way.

Of one preparation made from a great number of extracts the melting point of the crystallized picrate was 142°—143° and that of the piperidine picrate from synthetic piperidine (Heyl & Co) in the same way was 144°—145°. A mixture of equal parts of urine picrate and piperidine picrate melted at 143°—144°. Determination of the picric acid content and base-nitrogen showed a good agreement between base-picrate and piperidine picrate.

b) *Biological action of isolated pressor substance.*

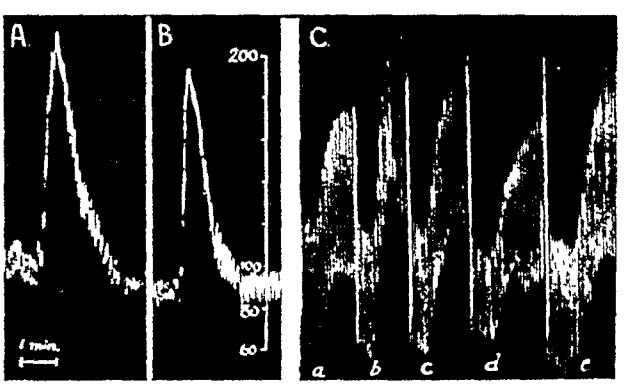
Though the active principle was tested regularly during the course of purification against piperidine picrate as a standard

and finally found to equate this compound weight for weight with regard to the action on the cat's blood pressure and on the isolated intestine of the rabbit, it was deemed desirable to test the relative effects also in other respects. The picrate could be tested without difficulties or obvious side effects in many cases, but for most of the tests the picrate was turned into hydrochloride or sulphate and tested against the corresponding salts of pure piperidine.

The pharmacological properties of piperidine have been investigated by several authors since MOORE and ROW (1897) made their thorough study, which clearly showed the close resemblance in action between piperidine and nicotine.

The isolated active substance from human normal urine was tested as to its action on the isolated intestine of the fowl, on the cat's pupil, on the respiration of the cat and dog, and in the unanaestetized frog. In all these instances the action of the isolated active substance as hydrochloride was identical with that of piperidine hydrochloride.

It could also be shown that the active extracts of human urine from various sources (non-smokers) showed the same colour reaction as given by piperidine with sodium-β-naphtho-quinone-4-sulfonate. The quantitative agreement between the figures obtained from the colorimetrical and biological tests when com-



0.8 mg pip HCl	30 ml 189 c	0.5 mg pip HCl	30 ml 189 c	0.5 mg pip HCl	15 ml 189 c	0.5 mg pip HCl

Fig. 1. A and B, blood pressure cat, chloralose. A. 0.8 mg piperidine hydrochloride, B. extract of 30 ml urine healthy male nonsmoker (U. R.) C. Isolated rabbit's intestine. a, c, and e 0.5 mg piperidine hydrochloride. b. extract of 30 ml urine, d, extract of 15 ml urine as in B

Table 1.

Number	Mg piperidine hydrochloride per 100 ml urine	
	Blood pressure cat	Colorimetrically
173	1.9	1.7
182	0.8	1.0
184	1.1	1.1
189 b	2.5	2.5
189 c	2.3	2.4
191 a	1.4	1.2
198 a	1.8	1.6

pared with piperidine hydrochloride was good, as shown by the following table 1, which also includes some clinical cases.

In smokers' urine the colorimetrically found amount was regularly less than the biologically found as might be expected since the biological activity of piperidine is only 1/15—1/20 of that of nicotine.

The close agreement between the biological and the colorimetrically found activity in terms of piperidine, in conjunction with the chemical data seem to warrant the conclusion that the pressor activity in normal urine chiefly consists of piperidine.

Summary.

Normal human urine from non-smokers contains piperidine up to 10 mg and more per litre, corresponding to a nicotine activity of about 0.5 mg per litre. After ether extraction and steam destillation the piperidine could be isolated as crystalline picrate, as previously demonstrated for cow's urine (Euler, 1944).

The pressor activity of ether extracts of normal human urine is chiefly due to piperidine as shown by a comparison of the biological action and the colorimetrically determined piperidine in urine extracts.

Urine from horse, pig, cat and rabbit contains piperidine in similar amounts as in human urine.

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Addendum. After this paper had been written, two articles in J. Physiol., 1944, 103, pp. 68 and 185 by MARY F. LOCKETT became available in this country. The activity of her preparations of male urine (tobacco use not excluded) corresponded to about 50—70 μ g of nicotine and in female urine to 17 μ g of nicotine base per litre. In our extracts, the total nicotine-like activity corresponded to some 300 μ g nicotine per litre on an average in non smokers' urine.

ACTA PHYSIOLOGICA SCANDINAVICA
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AUS DEM MEDIZINISCH-CHEMISCHEN INSTITUT DER UNIVERSITÄT LUND

OXYDATION ZYKLISCHER VERBINDUNGEN DURCH VITAMIN C

UNTER BESONDERER BERÜCKSICHTIGUNG DER ENTSTEHUNG VON UROCHROM A

VON

BIRGER EKMAN

LUND

1 9 4 4

Meiner Frau

VORWORT.

Die vorliegende Arbeit ist am Medizinisch-Chemischen Institut der Universität Lund in den Jahren 1939—1943 ausgeführt worden. Dem Vorstand des Instituts, Herrn Professor Dr. Erik Widmark sage ich meinen ehrerbietigen Dank für die guten Arbeitsbedingungen, für sein Interesse und für wertvolle Kritik.

Dozent Dr. Carl G. Holmberg hat meine Arbeit mit stetem Interesse verfolgt. Hierfür und namentlich für seine wertvollen Ratschläge spreche ich ihm meinen wärmsten Dank aus.

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Die freundliche Überreichung von Hormonpräparaten verdanke ich Igefa Svenska A/B und von beträchtlichen Askorbinsäuremengen Roche-Produkter AB, Stockholm.

Lund, im Januar 1944.

Birger Ekman.

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Einleitung.

In früheren Arbeiten (Ekman 1940, 1941 und 1942) hat der Verfasser zeigen können, dass die Ausscheidung von Urochrom A im Harn sowohl durch die Zufuhr von Askorbinsäure als durch die Zufuhr zyklischer Verbindungen beeinflusst wird, und ferner, dass Askorbinsäure in vivo und in vitro zyklische Verbindungen unter Bildung von Urochrom-A-ähnlichen Farbstoffen umwandelt.

Die Versuche, in denen gezeigt wurde, dass sich zyklische Verbindungen in vitro durch Askorbinsäure umwandeln lassen, fussten auf der Arbeit von Edlbacher und v. Segesser (1937 a), in welcher diese Forscher zu dem Ergebnis gekommen waren, dass Imidazolverbindungen durch Askorbinsäure oxydativ desaminiert werden. Ein gemeinsamer Zug der von mir untersuchten Reaktionen zwischen zyklischen Verbindungen und Askorbinsäure war indessen die Entstehung von Farbstoffen, die sich mit einer Urochrom-A-Bestimmungsmethodik (siehe unten) bestimmen liessen. Diese Farbstoffbildung blieb völlig oder fast ganz aus, wenn die Askorbinsäure auf aliphatische Verbindungen einwirkte. Zur Beleuchtung dieser Verhältnisse diene Tab. 1, in der die Farbstoffbildung, ausgedrückt durch die Extinktionswerte bei der Bestimmung von Urochrom A, bei Umwandlungsversuchen mit Alanin und Glykokoll sowie mit zyklischen Derivaten dieser Aminosäuren und mit einer einfachen aromatischen Verbindung verglichen wird.

In einer Reihe von Arbeiten konnte indessen Abderhalden (1934—1938) zeigen, dass sowohl zyklische als aliphatische Aminosäuren in ähnlichen Versuchen wie den von Edlbacher und v. Segesser durchgeführten eine Umwandlung erfahren, die von Abderhalden folgendermassen definiert wird: (die An-

TABELLE 1.

Vergleich zwischen der Farbstoffbildung bei Umwandlung durch Askorbinsäure von einigen aliphatischen Aminosäuren, von zyklischen Derivaten dieser Säuren, sowie von einer einfachen aromatischen Verbindung.

Kolbenversuche (siehe Kap. II). Die Farbstoffbildung wird mit dem Extinktionswert bei Urochrom-A-Bestimmung angegeben (siehe Kap. II). Zusatz in sämtlichen Versuchen: 352 mg Askorbinsäure, 13,6 mg Ferrizitrat. Für jede untersuchte Verbindung ist das molare Konzentrationsverhältnis zu Askorbinsäure und Ferrizitrat a) 1 : 2 : 0,05, b) 2 : 2 : 0,05. pH 5,8. Versuchszeit 18 Stdn.

	Alanin		Glykokoll		Phenylalanin		Histidin		Hippursäure		Phenol	
	a	b	a	b	a	b	a	b	a	b	a	b
Zusatz in mg	89	178	75	150	165	230	155	310	179	358	94	188
Urochrom A E.	0,12	0,12	0,13	0,17	0,67	1,0	0,92	1,6	0,80	0,88	0,67	0,75

nahme wird bestätigt, dass) »Ascorbinsäure bzw. die aus ihr sich bildende Dehydroascorbinsäure bei Anwesenheit von Sauerstoff (und von Eisen) aus Monoaminokarbonsäuren unter Ammoniak- und Kohlensäureabspaltung die um ein Kohlenstoffatom ärmeren Aldehyde hervorgehen lässt».

Nach Holtz (1936 a) kann Askorbinsäure als ein Oxydationskatalysator für ungesättigte Fettsäuren auftreten, und (1936 b) sie übt eine »Induktionswirkung» auf die Oxydation von Zucker aus.

Parrod (1938) gibt an, dass bei Oxydation von Askorbinsäure in Anwesenheit von Ammoniak oder primären Aminen sich Oxamide bilden.

Leibowitz und Guggenheim (1938) zeigten, dass Kaliumzyanid in vitro durch Askorbinsäure entgiftet wird.

Askorbinsäure besitzt also die ausgesprochene Fähigkeit, in vitro Verbindungen von ganz unterschiedlicher Zusammensetzung anzugreifen.

Es erschien von beträchtlichem Interesse, zu untersuchen, ob irgendwelche von diesen Askorbinsäurereaktionen auch physiologische Bedeutung haben, sowie gegebenenfalls den chemischen Verlauf eingehender zu studieren. Das Untersuchungsmaterial

musste dabei zwangsläufig beschränkt werden, und im Hinblick auf das charakteristische Auftreten gewisser Farbstoffe bei der Umwandlung zyklischer Verbindungen, sowie in Anbetracht dessen, dass diese sich wahrscheinlich leichter quantitativ bestimmen liessen, sind die Untersuchungen in der vorliegenden Arbeit auf gewisse zyklische Verbindungen beschränkt worden.

KAP. I.

Schrifttumsübersicht und Fragestellung.

Das Urochrom ist in der neueren Forschung nur geringem Interesse begegnet. Es erscheint daher angebracht, das einschlägige Spezialschrifttum einer verhältnismässig ausführlichen Durchsicht zu unterziehen.

Sehr umfangreich sind dagegen die Forschungen über das Vitamin C. In dieser Schrifttumsübersicht muss ich mich darauf beschränken, nur auf solche Arbeiten einzugehen, die das Vitamin C mit zyklischen Verbindungen oder mit Pigmentierung und Farbstoffbildung in Zusammenhang bringt.

I. Urochrom.

Seit langem teilt man die Harnfarbstoffe in zwei Fraktionen ein. Einmal diejenigen, die sich mit Ammoniumsulfat aussalzen lassen und gewöhnlich in Äther löslich sind. Zum andern die, welche auch dann in Lösung bleiben, wenn der Harn mit Ammoniumsulfat gesättigt wird, die aber zum grösseren Teil in Äther unlöslich sind. Die letzteren hat man Urochrom genannt und sie im allgemeinen als einen einheitlichen Farbstoff betrachtet, der dem Harn die charakteristische Färbung gebe.

Es ist indessen klar, dass der Begriff des Urochroms, da er eigentlich nur bedeutete, dass es sich um einen im Harn vorkommenden Farbstoff mit der negativen Eigenschaft, nicht mit Ammoniumsulfat ausgesalzt werden zu können, handelte, sich auf mehrere verschiedene Verbindungen ausgedehnt hat. Trotzdem hat man die Bezeichnung beibehalten.

Eine ausführliche Zusammenstellung des älteren Schrifttums

findet sich bei NEUBAUER-HUPPERT (1913), und von den älteren Arbeiten sei hier nur kurz auf die von GARROD, DOMBROWSKI, HOHLWEG und WEISS eingegangen, auf denen die neueren Untersuchungen weitgehend zu fussen scheinen.

GARROD (1894) isolierte sein Urochrom in der Weise, dass er den Harn durch Sättigung mit Ammoniumsulfat zunächst von anderen Farbstoffen befreite und dann durch Zusatz von Alkohol zum Filtrat eine urochromhaltige Alkoholschicht erhielt. Die Lösungsverhältnisse waren dieselben wie bei DOMBROWSKIS Urochrom (siehe unten). Eine Elementaranalyse hat GARROD nicht vorgenommen, doch gab er an, der Farbstoff sei stickstoffhaltig. GARROD war der Meinung, er könne das Urochrom, indem er es mit Azetaldehyd behandle, in Urobilin verwandeln, und nahm demgemäss an, die Gallfarbstoffe seien das Ausgangsmaterial der Urochrombildung.

DOMBROWSKI (1908) isolierte sein Urochrom, indem er es mit Kupferazetat aus einem Harn ausfällte, der vorher von Sulfaten und Phosphaten befreit worden war. Bei quantitativen Bestimmungen arbeitete DOMBROWSKI teils mit Fällung des Urochroms mittels Kupferazetat und anschliessender Stickstoffanalyse der Fällung, teils mit einem Verfahren, das sich auf die Fähigkeit des Urochroms gründet, Jodsäure unter Abscheidung von Jod zu reduzieren. Die beiden Methoden lieferten übereinstimmende Ergebnisse.

Dieses Urochrom konnte eine Lösung von Ferrizyankalium-Ferrichlorid unter Hervorbringung von Berlinerblau reduzieren. Es war in Wasser leichtlöslich, in absolutem Alkohol schwerlöslich, in 90 % Alkohol löslich und konnte mit Äther aus der alkoholischen Lösung gefällt werden. Es reagierte sauer. Die Elementaranalyse zweier Präparate hatte folgendes Ergebnis: C: $45{,}_{32}$ $43{,}_{42}$; H: $5{,}_{26}$ $5{,}_{33}$; N: $9{,}_{49}$ $10{,}_{78}$; S: $5{,}_{59}$ $5{,}_{89}$; O: $34{,}_{34}$ $34{,}_{58}$.

Die von normalen Versuchspersonen pro Tag ausgeschiedene Menge betrug $0{,}_{37}$—$0{,}_{69}$ g. Verabfolgte man eine reine »Milchkost» (Milch und Kartoffeln), so nahm die Ausscheidung ab, ging man zu fleischreicher Diät über, so stieg die Ausscheidung. Unter krankhaften Verhältnissen war die Ausscheidung gesteigert, besonders bei Typhus und Leberzirrhose. DOMBROWSKI betrachtete das Urochrom als ein Eiweissabbauprodukt.

Durch Kochen mit Säuren konnte das Urochrom in ein Melanin umgewandelt werden.

Hohlweg (1908) isolierte ein Urochrom, indem er aus einem Harn, der durch Fällung mit Chlor-Kalzium in alkalischem Milieu von den übrigen Farbstoffen befreit worden war, das Urochrom an tierische Kohle adsorbierte und dann mit Eisessig eluierte. Die Löslichkeitsverhältnisse seines Präparats stimmen mit denen des Dombrowskischen Urochroms überein.

Die Elementaranalyse zeigte indessen, dass es keinen Schwefel enthielt: C: 47,58 %, H: 6,30 %, N: 9,89 %.

Hohlweg nahm an, dass der Schwefelgehalt in Dombrowskis Urochrom eine Verunreinigung gewesen sein müsse.

Ein gemeinsamer Zug dieser Urochrompräparate ist, dass sie sehr leicht zerstört werden und grosse Reaktionsbereitschaft zeigen.

Garrods, Dombrowskis und Hohlwegs Urochrome wurden im allgemeinen, trotz der hinsichtlich des Isolierungsverfahrens und der Zusammensetzung bestehenden Unterschiede, als ein und derselbe Stoff betrachtet, und als der Stoff, der dem Harn hauptsächlich seine Farbe verleiht.

Dies bestritt indessen Weiss (1911). Er meinte, Dombrowskis Urochrom habe keine grössere Bedeutung für die Färbung des Harns. Diese sei vielmehr durch ein Urochromogen bedingt, das sich durch leichte Oxydation (z. B. mit Kaliumpermanganat) in ein »echtes» Urochrom umwandeln lasse. Sowohl das Dombrowskische als das Weisssche Urochrom gehören s. E. der »Proteinsäurefraktion des Harns» an, d. h. den Verbindungen, die in Wasser lösliche, durch Alkohol fällbare Barytsalze bilden. Weiss hielt sein Urochromogen für den Träger der Ehrlichschen Diazoreaktion im Harn.

Bei quantitativen Bestimmungen beseitigte Weiss störende Farbstoffe mit Ammoniumsulfat und verglich dann die Farbe des Filtrats mit einer Lösung von Echtgelb, teils direkt, teils nach Zusatz von Kaliumpermanganat. Im ersteren Falle erhielt er einen Ausdruck für den ursprünglichen Urochromgehalt, im letzteren einen Wert für den ursprünglichen Urochromgehalt einschliesslich des aus dem Urochromogen gebildeten Urochroms. Das Verfahren lieferte nur relative Werte. Die Werte des Urochromogens ändern sich nach Weiss stets parallel zur Stärke der Ehrlichschen Diazoreaktion.

Weiss erklärt, sein Urochrom und Urochromogen unterschieden sich deutlich von Dombrowskis mit Kupferazetat ausfällbarem Urochrom. Beide können mit Bleiazetat gefällt werden, doch löst sich Dombrowskis Urochrom bei Zusatz verdünnter Essigsäure, während das Weisssche Urochrom in Fällung verbleibt.

Die Arbeiten der obengenannten Autoren begründeten eine umfassende Urochromforschung, die indessen recht widersprechende Ergebnisse gezeigt hat, da nach wie vor keine einheitlichen Substanzen haben untersucht werden können. Hier sei nur auf diejenigen Arbeiten eingegangen, die durch eine definierte Methodik oder durch Analyse der isolierten Präparate einen Vergleich mit dem durch Kupferazetat ausfällbaren Urochrom erlauben, das in der vorliegenden Arbeit abgehandelt wird.

Pelkan (1920) bestimmte teils die totale Harnfarbe, teils die Restfarbe nach Behandlung des Harns mit Bleiazetat, welch letztere »contained urochrome as the principal coloring matter», und konnte feststellen, dass beide Farbfraktionen durch den Proteingehalt der Nahrung beeinflusst werden. Er sah, dass eine verminderte Eiweisszufuhr eine starke Farbverminderung zur Folge hatte, während hingegen vermehrter Eiweissverzehr nur eine verhältnismässig viel geringere Farbverstärkung bewirkte. Indessen stellte es sich heraus, dass, um Farbveränderungen hervorzurufen, solche Proteine verwendet werden mussten, die zyklische Aminosäuren enthielten.

Roar (1921) konnte mittels einer auf dem Garrodschen Isolierungsverfahren fussenden Methode zeigen, dass Kaninchen, wenn sie chlorophyllhaltige Pflanzenteile verabreicht bekommen, grosse Mengen eines Farbstoffs ausscheiden, der nicht mit Ammoniumsulfat gefällt werden kann und der in Alkohol löslich ist. Roar nimmt auf Grund dieser Untersuchungen an, dass es sich bei dem Urochrom um ein Chlorophyllderivat handelt.

Bei Untersuchungen über die Ursache der Ehrlichschen Diazoreaktion im Harn konnten Hermanns und Sachs (1921) sowie Hermanns (1922) zeigen, dass man es hier nicht mit einem einheitlichen Stoff zu tun hat. Früher versuchte man im allgemeinen, Stoffe zu isolieren, welche die Ehrlichsche Reaktion auslösten, diese Forscher aber isolierten statt dessen die Farbstoffe, die durch Koppelung an das Ehrlichsche Reagens entstanden. Sie konnten zeigen, dass ein solcher Farbstoff im Harn eines Patienten mit Leberkrebs eine Oxyindolessigsäure enthielt, während ein Diazofarbstoff aus dem Harn eines Tuberkulösen eine Verbindung enthielt, die man als ein mehrwertiges Phenol (wahrscheinlich ein im Benzolkern oxydiertes Kumaron) ansprechen zu können glaubte, das bei Hydrolyse Schwefelsäure lieferte. Sie kamen zu dem Schluss, dass »das Wesen der Ehrlichschen Diazoreaktion auf der Ausscheidung phenolartiger Stoffwechselprodukte beruht und sicherlich anzusehen ist als der Ausdruck eines toxischen Eiweisszerfalls».

Weiss hat in zahlreichen Arbeiten seine Untersuchungen über das Urochrom fortgesetzt, sich dabei aber fast ausschliesslich für die Be-

ziehungen desselben zur EHRLICHschen Diazoreaktion interessiert. Er hält an seiner alten Anschauung fest, dass die Ursache dieser Reaktion das Urochromogen sei. Hier soll nur auf die Untersuchungen eingegangen werden, die den Eigencharakter des Urochroms und des Urochromogens berühren.

In einer Arbeit (1920 a) legt WEISS ein Verfahren zur Fraktionierung der Farbstoffe des Harns vor. Dieses besteht in der Ausfällung einer »Urobilinfraktion» mit Hilfe von Bleiazetat. Durch Zusatz von Lauge zum Filtrat bekommt man eine in verdünnter Essigsäure lösliche Fällung, die aus einer »Urochromfraktion» besteht.

Diese Urochromfraktion dunkelt nach, und zwar infolge der Anwesenheit von Urochromogen, das über Urochrom zu einem Melanin werden kann. Dieses Nachdunkeln, das besonders in alkalischem Milieu hervortritt, will WEISS durch die Annahme erklären, dass die Farbstoffe ein Polyphenolderivat enthalten. Die Urochromfraktion ist stickstoffhaltig, löst aber keine ADAMKIEWICZ-LIEBERMANNsche Reaktion aus. Sie kann nicht mit Ammoniumsulfat ausgesalzt werden. Das Melanin und damit auch Urochrom und Urochromogen sind schwefelhaltig — in einer späteren Arbeit (1923) beziffert WEISS den Schwefelgehalt des Melanins auf 3,5 % organisch gebundenen Schwefel. Nach WEISS (1920 b) besteht kein Zusammenhang zwischen der Kost und der Urochromausscheidung, sondern das Urochrom ist als »intermediäre, ausschliesslich dem Gewebszerfall entstammende Stoffwechselprodukte» aufzufassen. Das Ausgangsmaterial der Urochrombildung sind nach WEISS' Ansicht Phenylalanin und Thyrosin oder Derivate dieser Stoffe, und als Bildungsort betrachtet er die Nieren.

FISCHER und ZERWECK (1924) isolierten Urochrom aus 1600 Liter Harn nach einem Verfahren, das sich von dem im allgemeinen angewandten unterscheidet. Der Harn wurde im Vakuum eingedampft, und von den sich bildenden Kristallmassen wurde ein sirupähnliches Konzentrat abgesaugt. Das Konzentrat wurde durch Dialyse gereinigt, und nach vollständiger Dialyse konnte der Farbstoff durch Zusatz von Säure in Flocken ausgefällt werden. Auf diese Weise wurden 50 g Urochrom isoliert. Die Elementaranalyse hatte folgendes Ergebnis: C 52 %, H 6 %, N 11 %, S 2 %. Der Farbstoff konnte nicht in kristallinischer Form dargestellt werden. Er löste keine Pyrrolreaktion aus und die EHRLICHsche Reaktion war negativ. Bei der Hydrolyse zeigte es sich, dass er Thyrosin, Histidin und Arginin enthielt. Die Verfasser sind der Meinung, dass er als ein Eiweissabkömmling aufzufassen sei, der gewisse Ähnlichkeiten mit den Oxyproteinsäuren habe und wahrscheinlich vom Muskelfarbstoff stamme. Die Analysen legen den Schluss nahe, dass es sich um einen Stoff von komplizierter Zusammensetzung handelt, und die Autoren machen auch gewisse Vorbehalte in bezug auf die Einheitlichkeit geltend: »Natürlich ist der Einwand nicht von der Hand zu weisen, dass der färbende Bestand-

teil etwas anderes ist, etwa als Verunreinigung im Eiweissabkömmling angehäuft ist.»

POLLECOFF (1924) meinte zeigen zu können, dass der Farbstoff, der nach GARROD und WEISS isoliert werden kann, Glykuronsäure enthält.

DRABKIN (1927) gab eine Methode zur Bestimmung von Urochrom an, die ganz einfach aus einem direkten Kolorimetrieren des gut gefilterten Harns besteht. Als Beweis dafür, dass die totale Harnfarbe ausschliesslich durch das Urochrom bestritten werde, diente ihm folgender Versuch: Der Harn wird mit Kalziumoxyd und Ammoniumsulfat behandelt. Es entsteht ein Niederschlag, der bei normalen Harn aus etwa 30 % der Gesamtfarbe besteht. Der Niederschlag wird mit WASSER gewaschen, dieses wird eingedampft, und legt man den Rest dann zu dem ursprünglichen Filtrat, so erhält man auch die ursprüngliche Farbstärke zurück. Der Verfasser folgerte daraus, dass der erhaltene Niederschlag nur mitgerissenes Pigment und nicht das Produkt einer wirklichen Fällung war; dass der Harn also nur mit Ammoniumsulfat nicht fällbare Farbstoffe, d. h. Urochrom enthalte.

Mit dieser Methode fand DRABKIN, dass die Urochromausscheidung bei Fasten, bei Zufuhr von Salzsäure, bei BASEDOWscher Krankheit, Diabetes und Typhus zunimmt.

In einer späteren Arbeit meint DRABKIN (1930) dieses Urochrom mit n-Butylalkohol bei einem pH von etwa 4 extrahieren zu können. Es liegen keine Beweise dafür vor, dass dieser isolierte Farbstoff allein die Farbe des Harns bewirkte, d. h. nach DRABKIN Urochrom wäre, doch ist der Farbstoff einer eingehenden Untersuchung unterzogen worden, die von Interesse ist. Die Löslichkeitsverhältnisse stimmen mit den für das DOMMOWSKIsche Urochrom gefundenen überein. Es ist schwach reduzierend, kann aber Jodsäure nicht reduzieren. Eine SH-Gruppe war nicht nachzuweisen, doch wurde der Schwefelgehalt mit 0,61 % ermittelt. Der Farbstoff lieferte negative Biuretreaktion, schwach positive ADAMKIEWICZ-Reaktion, positive MILLON-Reaktion. Er kann durch Reduktionsmittel entfärbt werden und dann seine Farbe durch Zusatz von Wasserstoffsuperoxyd zurückgewinnen. Eine chromatographische Untersuchung ergab, dass es sich um einen einheitlichen Farbstoff handeln dürfte. Die Ausscheidung pro Tag belief sich bei normalen Individuen auf 73 mg.

In einer Arbeit über den Zusammenhang zwischen Tryptophan und Urochrom referieren KOTAKE und SAKATA (1930) Untersuchungen von TANI über den Effekt der Zufuhr von Thyrosin, Phenylalanin, Histidin, Cystin und Tryptophan auf die Ausscheidung von Urochrom, das nach dem WEISSschen Verfahren bestimmt wurde. Mit Tryptophan erzielte TANI eine starke Steigerung, während die übrigen Aminosäuren keine Wirkung hatten. KOTAKE und SAKATA wiederholten die Versuche mit Tryptophan und machten ausserdem Versuche mit einem Zerfallsprodukt dieses Stoffes, dem Kynurenin, und konnten die Befunde TANIS sowohl im Tierversuch (Kaninchen) als im Selbstversuch bestätigen. Die Uro-

chrombestimmungen machten sie sowohl nach WEISS als nach DOMBROWSKIS Jodmethode, ausserdem wurden die Diazowerte nach EHRLICH verfolgt. Alle Verfahren lieferten übereinstimmende Ergebnisse.

BARRENSCHEEN und DELL' ACQUA (1930) konnten zeigen, dass die reduzierende Fähigkeit des normalen Harns auf gewissen Farbstoffen beruht, die sich durch eine Methylalkohol-baryt-Lösung als ein Bariumsalz aus ihrer alkoholischen Lösung ausfällen lassen. Die Eigenschaften der isolierten Farbstoffe stimmen »im Wesentlichen mit den Eigenschaften des Urochroms von DOMBROWSKI» überein. Zur quantitativen Bestimmung dieser Harnfarbstofffraktion bedienen sich diese Autoren der Fähigkeit der Farbstoffe, eine Ferrichlorid-Ferrizyanidlösung zu Berlinerblau zu reduzieren, und sie legen diese Eigenschaft einem kolorimetrischen Verfahren zugrunde. Es zeigte sich, dass die Farbstoffe freie SH-Gruppen enthielten, und durch Blockieren derselben mit Formaldehyd konnte gezeigt werden, dass die Reduktionsfähigkeit sowohl durch SH-haltige als auch andere Farbstoffe bedingt war. Auch für die quantitative Bestimmungsmethodik liess sich eine solche Fraktionierung durchführen. Bei Untersuchungen am Menschen zeigte es sich, dass die Ausscheidung beider Fraktionen im Harn durch Verabfolgung einer eiweissarmen, vegetabilischen Kost gesenkt und durch eiweissreiche, vegetabilienarme Kost gesteigert werden konnte. Im letzteren Falle wurde ganz besonders die Ausscheidung der SH-haltigen Fraktion gesteigert. Die Autoren nahmen an, dass es sich bei der SH-haltigen Fraktion um Urochrom handle. Rechnete man die Reduktionswerte für diese Fraktion mit Hilfe der DOMBROWSKIschen Prozentzahlen für Schwefel in Urochrom um und verglich mit den Urochromwerten, die in demselben Harn mit DOMBROWSKIS Jodsäureverfahren gefunden wurden, so ergab sich eine sehr gute Übereinstimmung.

Die nicht sulfhydrylhaltige Fraktion sprach man als ein Polyphenol an, eine endgültige Erklärung seiner Zusammensetzung konnte man nicht geben.

Erneute Untersuchungen der Harnfarbstoffe machten dann HEILMEYER und seine Mitarbeiter (1930 und 1934) unter Verwendung des ZEISSschen PULPHRICHphotometers. HEILMEYER unterscheidet zwischen nicht mit Ammoniumsulfat fällbaren Farbstoffen (Fraktion I), Urochrom A, und mit Ammoniumsulfat fällbaren Farbstoffen (Fraktion II), Urochrom B, Urobilin und Uroerythrin. Urochrom A bestreitet etwa 35 % der normalen Harnfarbe und entspricht dem WEISSschen Urochrom. Zum Unterschied von WEISS bestreitet aber HEILMEYER jeden Zusammenhang des Urochrom A mit der EHRLICHschen Diazoreaktion, und ferner weist er darauf hin, wie unspezifisch die WEISSsche

Permanganatprobe sei. Weiter kann HEILMEYER zeigen, dass eine Bleiazetatfällung anderer Farbstoffe als Urochrom A mengenmässig ganz von der Menge des Fällungsmittels und von der Menge der Phosphate im Harn abhängig ist. Das Filtrat zur quantitativen Urochrombestimmung zu verwenden (wie es z. B. PELKAN getan hat), kann also nicht zuverlässige Resultate geben.

MIWA (1935) gibt an, die WEISSsche Urochromfraktion sei stets mit Glykose, aromatischen Oxysäuren, Harnsäure und Brenzkatechinester verunreinigt. Dieser letztere liefert bei Hydrolyse Brenzkatechin, was die EHRLICHsche Diazoreaktion auslösen kann. MIWA meint, dass das Brenzkatechin den von HERMANNS und SACHS als Ursache der Diazoreaktion vermuteten phenolartigen Stoffwechselprodukten entspreche. Von der WEISSschen Urochromfraktion war angegeben, sie löse eine Pyrrolreaktion aus. MIWA ist der Meinung, dass diese durch einen Indolkörper bedingt wird, der auch die Diazoreaktion auslösen könne. Durch das Vorkommen sowohl eines Brentzkatechinesters als einer Indolverbindung, die beide ebenso wie das Urochromogen als solches die Diazoreaktion herbeiführen können, erklärt es sich, dass die Diazoreaktion nicht immer parallel zur Urochromogenmenge verläuft. Die Urochromogenausscheidung sei besonders bei fieberhaften Krankheiten gesteigert.

Stellt man die WEISSsche Urochromfraktion dar, so erweist sie sich als schwefelfrei.

RANGIER (1935) isolierte ein Urochrom, indem er in schwach essigsaurem Milieu Harn mit tierischer Kohle schüttelte und dann mit verdünntem Ammoniak eluierte. Das Eluat wurde durch Dialyse gereinigt, und nach Eindampfen blieb ein amorphes Urochrompräparat zurück. Die Elementaranalyse ergab folgende Zusammensetzung: C 51 %, H 5,9 %, N 12 %, S 2 %. Der Farbstoff war in Wasser und verdünntem Alkohol leichtlöslich, »in den gebräuchlichen organischen Lösungsmitteln« aber unlöslich. Er konnte durch Zusatz von Natriumhydrosulfit entfärbt werden und gewann seine Farbe zurück, wenn man ihn an der Luft stehen liess.

(Der isolierte Farbstoff konnte eine komplexe Verbindung mit Harnsäure eingehen, deren Wasserlöslichkeit hierdurch erheblich gesteigert wurde.)

1938 fanden RANGIER und TRAVERSE, dass man durch Hydrolyse des in der oben angegebenen Weise isolierten Urochroms mit verdünnter Schwefelsäure eine kristallisierende Substanz erhalten konnte, die sich als Indoxyl erwies. Bei fortgesetzten Untersuchungen (1939) konnten sie zeigen, dass ihr Urochrompräparat auch Glykuronsäure enthielt. Sie nahmen an, dass das Urochrom aus Polypeptidketten aufgebaut ist, die durch Glykuronsäure an Indoxyl gebunden sind.

Bei einer Durchsicht des vorstehend referierten Schrifttums fällt es auf, dass die Autoren, die quantitative Bestimmungen

der Urochromausscheidung im Harn gemacht haben, diese im allgemeinen mit dem Eiweissstoffwechsel in Verbindung bringen und sie in mehreren Fällen sogar durch Einverleibung verschiedener Eiweissstoffe oder zyklischer Aminosäuren mit der Kost beeinflussen konnten.

Man hat mehrere der quantitativen Bestimmungsmethoden kritisiert, und zwar sowohl aus dem Gesichtspunkt, dass die Methodik Urochromverluste mit sich bringe, als auch aus dem entgegengesetzten, nämlich dass die Methodik nicht spezifisch sei. Wenn trotzdem im ganzen übereinstimmende Resultate erzielt werden konnten, so lässt sich dies vielleicht dadurch erklären, dass das Urochrom wirklich der »Hauptfarbstoff» des Harns ist und auf diese Weise die Resultate trotz der Mängel der angewandten Methoden beherrschen konnte.

Auch vom qualitativen Gesichtspunkt aus lassen sich mehrere gemeinsame Eigenschaften bei den untersuchten Urochrompräparaten feststellen, selbst wenn sich diese anscheinend in wesentlichen Punkten voneinander unterscheiden können. Versucht man, aus den vorliegenden Untersuchungen ein »Durchschnittsurochrom» herauszuschälen, so dürfte dieses folgende Eigenschaften haben:

1. Es ist mit Kupferazetat und alkoholischer Barytlösung fällbar. Es kann an tierische Kohle adsorbiert werden. Es lässt sich nicht mit Ammoniumsulfat oder Bleiazetat fällen.

2. Es ist in Wasser leichtlöslich, in 90 % Alkohol löslich, in absolutem Alkohol schwerlöslich, in Äther unlöslich.

3. Es reagiert sauer.

4. Es hat reduzierende Eigenschaften, wahrscheinlich bedingt durch SH-Gruppen, Glykuronsäure oder Oxydationsprodukte von Phenolen.

5. Es ist stickstoffhaltig.

6. Seine eventuelle Fähigkeit, mit dem EHRLICHschen Reagens Diazofarbstoffe zu bilden, dürfte auf Brentzkatechin oder anderen Phenolabkömmlingen sowie Indoxyl beruhen, die in dem Urochrommolekül enthalten sind.

7. Es kann durch Reduktionsmittel in eine farblose Form (einem »Urochromogen» entsprechend) gebracht und dann durch Oxydationsmittel in die farbige Form zurückgeführt werden.

Es ist in keinem Falle gelungen, ein kristallinisches Präparat darzustellen, und nur in einem Falle (DRABKIN) ist versucht worden festzustellen, ob ein einheitlicher Farbstoff vorgelegen hat oder nicht. Es erscheint naheliegend, dass die Unterschiede hinsichtlich Eigenschaften und Zusammensetzung, die in gewissen Fällen zwischen den Präparaten der einzelnen Forscher bestehen, durch das Vorhandensein von Verunreinigungen erklärt werden können. Dies kann z. B. die Erklärung für die sich widersprechenden Angaben betreffend den Schwefelgehalt des Urochroms sein.

Eine weitere Möglichkeit zur Erklärung der Unterschiede in den Eigenschaften dürfte darin gesehen werden können, dass das Urochrom verschiedene Oxydationsstufen aufweisen zu können scheint und dass unterschiedliche Isolierungsverfahren mehr oder weniger vollständig oxydierte Präparate geliefert haben.

Zusammenfassend dürfte man annehmen können, dass es sich bei der Urochromforschung letztlich um ein und denselben Farbstoff gedreht hat, dass aber die Instabilität und das Vorkommen von Verunreinigungen in den isolierten Präparaten einer einheitlichen Anschauung betreffs der Eigenschaften des Stoffes hindernd im Wege gestanden haben.

In eigenen Versuchen (EKMAN 1940 und 1942) konnte gezeigt werden, dass die Ausscheidung von Urochrom A (Bestimmungsmethode siehe Kap. II) nach Zufuhr von Eiweissstoffen, Phenol und Thyroxin zunahm, dass aber Chlorophyll keine Einwirkung ausübte. Durch Einverleibung von Askorbinsäure im Überschuss sank die Urochrom-A-Ausscheidung, bei Skorbut und relativem C-Vitamin-Mangel stieg sie an.

Bei Studien über die Umwandlung zyklischer Verbindungen durch Askorbinsäure *in vitro* konnte gezeigt werden, dass diese Umwandlung unter Bildung von Farbstoffen erfolgte, die sich mittels des DOMBROWSKIschen Verfahrens isolieren liessen (S. 13). In vorläufigen Untersuchungen legten sie dieselben Eigenschaften an den Tag, die das nach demselben Verfahren aus Harn isolierte Urochrom kennzeichnen.

Wenn man von dem festgestellten Zusammenhang zwischen Urochrom-A-Ausscheidung und Zufuhr von Vitamin C absieht, stimmen also die Ergebnisse der Versuche in vivo mit denen

der früheren Autoren überein. Die Eigenschaften der in vitro gewonnenen Farbstoffe befinden sich ebenfalls im allgemeinen in Übereinstimmung mit den in den obigen Zusammenfassung gemachten Angaben. Gewisse von diesen Farbstoffen wurden jedoch bei Versuchen in vitro erhalten, in denen keiner der reagierenden Stoffe Stickstoff oder Schwefel enthielt, weshalb also auch diese Farbstoffe keines dieser Elemente enthalten können.

II. Vitamin C und Farbstoffbildung (Pigmentierung).

In Studien über den Oxydationsvorgängen bei Pflanzen machte Szent-Györgyi (1925, 1930, 1934) die Feststellung, dass *die charakteristische Farbe, die beschädigte Pflanzenteile zu* zeigen pflegen, ausbleibt, wenn das Gewebe Askorbinsäure enthält. Diese Beobachtung vergleicht er mit der Tatsache, dass man durch Einverleibung von Askorbinsäure bei Morbus Addison die charakteristische Pigmentierung zum Verschwinden bringen konnte.

Später erzielten Morawitz (1934) und Gutzeit (1935) einen Rückgang der im Zusammenhang mit krankhaften Zuständen des Magendarmkanals auftretenden Hautpigmentierung durch Einverleibung von Vitamin C. Bei Versuchen, experimentell einen Zusammenhang zwischen Askorbinsäure und Pigmentierung nachzuweisen, konstatierte Schade (1935), dass die Pigmentierung, die bei Bestrahlung mit einer Höhensonne-Lampe aufzutreten pflegt, bedeutend weniger stark ist, wenn die Versuchsperson vorher 10 Tage lang 200 mg Askorbinsäure täglich bekommen hat. In einem ähnlichen Versuch, wahrscheinlich aber mit kleineren Mengen Vitamin C, sah jedoch Drigalski (1934) ein negatives Resultat.

In diesem Zusammenhang ist zu erwähnen, dass Kahler und la Croix (1935) zeigen konnten, dass nach intensiven Sonnenbädern in mehr als der Hälfte der untersuchten Fälle im Harn eine positive »Melanogen-» Reaktion nach Thormählen zu verzeichnen war. Bei Zufuhr von Askorbinsäure (150 mg pro Tag) wurde die Reaktion negativ.

Man hat versucht, die Hemmung der Pigmentbildung in der Haut durch verschiedene Versuche in vitro zu beleuchten, und man ist dabei von der

Blochschen (1917) Theorie vom Dioxyphenylalanin (Dopa) als Pigmentmuttersubstanz und Pigmentbildung durch eine besondere Dopaoxydase ausgegangen. Schroeder (1934) und Grüneberg u. Schade (1934) konnten zeigen, dass die Dopareaktion (Dunkelwerden von Gewebsschnitten in einer Dopalösung) verhindert wurde, wenn man der Versuchslösung Askorbinsäure zusetzte. Gleichsinnige Ergebnisse erzielten Fantl und Fessler (1935), doch weisen sie darauf hin, dass die Askorbinsäure, um wirksam sein zu können, in einer Konzentration vorhanden sein muss, die unter physiologischen Bedingungen nur selten gegeben ist (über 2 mg%). Demgegenüber wäre indessen hervorzuheben, dass man bei Ausführung der Dopareaktion mit einer Dopakonzentration arbeitet, die ebenfalls nicht unter physiologischen Verhältnissen vorkommt (0,1 % Dopalösung).

Abderhalden (1934 und 1936) konnte zeigen, dass Askorbinsäure das Auftreten einer Rötung bei Einwirkung der Thyrosinase auf Thyrosin, 1-3-4-Dioxyphenylalanin und l-Adrenalin verhindert, dass dies aber wenigstens im Falle des Thyrosins nicht bedeutet, dass die Oxydation des Thyrosins verhindert wird: es war möglich, das Vorkommen von Dioxyphenylalanin nachzuweisen.

Auch die Bräunung, die in reinen Lösungen von Adrenalin und Dopa aufzutreten pflegt, konnte durch einen Zusatz von Askorbinsäure verhindert werden.

Schliesslich sind hier die auf S. 21 referierten eigenen Untersuchungen über das Urochrom A zu erwähnen. Bei Zufuhr von Askorbinsäure verminderte sich die Ausscheidung dieses Farbstoffs im Harn.

In den hier referierten Versuchen in vitro sind Farbstoffe aus aromatischen Verbindungen hervorgegangen, und dies kann auch bei den Untersuchungen in vivo der Fall gewesen sein. Die Hemmung der Farbstoff- und Pigmentbildung dürfte sich daher gemäss Szent-Györgyi (z. B. 1934) erklären lassen, welcher der Ansicht ist, dass bei der Oxydation aromatischer Verbindungen beispielsweise durch ein Phenoloxydasesystem Polyphenole gebildet werden. Durch die Anwesenheit der stark reduzierenden Askorbinsäure wird die weitere Oxydation zu Chinonen und damit die Farbstoffbildung verhindert, und statt dessen werden die Polyphenole zu Phenolen reduziert.

III. Vitamin C und zyklische Verbindungen.

In der Literatur finden sich zahlreiche Angaben über den Zusammenhang zwischen dem Abbau zyklischer Verbindungen und Vitamin C. Diese zyklischen Verbindungen weisen hinsichtlich ihrer chemischen Zusammensetzung und biologischen Bedeutung grosse Unterschiede auf. Der Übersichtlichkeit halber gliedere ich sie in folgende Gruppen:

1. Zyklische Aminosäuren.
2. Hormone.
3. Toxine.
4. Andere zyklische Verbindungen von pharmakologischem oder toxikologischem Interesse.

1. Zyklische Aminosäuren.

In der Einleitung wurde erwähnt, dass ABDERHALDEN gezeigt hat, dass Askorbinsäure die Fähigkeit hat, Monoaminokarbonsäuren in die entsprechenden um ein Kohlenstoffatom ärmeren Aldehyde umzuwandeln, und dass EDLBACHER und v. SEGESSER gefunden haben, dass Imidazolderivate bei Anwesenheit von Askorbinsäure eine oxydative Desaminierung erfahren.

ABDERHALDEN (1936) untersuchte in erster Linie Thyrosin und zeigte, dass bei Einwirkung von Askorbinsäure (oder, in geringerem Grade, auch von Dehydroaskorbinsäure), Sauerstoff der Luft und Eisen aus Thyrosin Dioxyphenylalanin gebildet wird. Der Versuch konnte mit Cystein oder Glutathion nicht wiederholt werden. In späteren Untersuchungen (1938) konnte er auch die Bildung von Dioxyphenylazetaldehyd nachweisen. Und bei Versuchen mit einer ganzen Reihe anderer Aminosäuren konnten die entsprechenden Aldehyde isoliert werden.

In einer anderen Arbeit zeigte ABDERHALDEN (1937), dass Histidin durch Askorbinsäure desaminiert wird und dass sich dabei ein rot-violetter Farbstoff bildet. Indessen konnte er keinen Aldehyd nachweisen. Farbstoffe traten auch bei anderen Aminosäuren auf, doch in bedeutend schwächerer Konzentration und im allgemeinen mit einem mehr gelblichen Farbton. Intensive

Farbstoffbildung sah er bei Phenylalanin, doch war der Farbton hier mehr braun.

EDLBACHER und v. SEGESSER (1937 a) untersuchten die Einwirkung von Askorbinsäure auf Imidazolverbindungen bei Anwesenheit von Luftsauerstoff und dreiwertigem Eisen und fanden, dass diese Imidazolverbindungen, vor allem aber Histidin und Histamin, eine oxydative Desaminierung erfuhren, die in einer Spaltung des Imidazolringes zum Ausdruck kam. Im Einklang mit ABDERHALDEN heben EDLBACHER und v. SEGESSER (1937 b) hervor, dass Eisen durch Hämoglobin ersetzt werden kann, dass dann aber auch das Hämoglobin angegriffen wird und ein grüner Farbstoff auftritt, der gewisse Gallfarbstoffreaktionen gibt. Die Autoren stellen fest, dass die Kombination Askorbinsäure-Hämoglobin-Sauerstoff ein Oxydationssystem darstellt und dass die Einwirkung auf das Histidin einer Histaminasewirkung zu vergleichen sei.

Gleichzeitig mit diesen Untersuchungen fand auch HOLTZ (1937), dass Histidin bei Sauerstoffzufuhr durch Askorbinsäure desaminiert werden kann. HOLTZ meint indessen, dass dann gleichzeitig eine Dekarboxylierung minderen Ausmasses auftrete, die zur Bildung von Histamin führe. Die Umwandlung des Histidins betrachten HOLTZ und TRIEM (1937 a) als eine Histidasewirkung, und sie zeigen, dass unter ähnlichen Verhältnissen dieselbe Wirkung auch mit Thioglykolsäure erzielt werden kann. In eingehenden Untersuchungen bestätigen HOLTZ und HEISE (1937 a und b), dass Histamin aus Histidin durch Einwirkung von Askorbinsäure, Glutathion, Cystein oder Thioglykolsäure bei Anwesenheit von Sauerstoff erhalten werden kann.

Sowohl ABDERHALDEN (1938) als EDLBACHER und v. SEGESSER (1937 c) geben indessen an, sie hätten in ihren Versuchen keine Histaminbildung nachweisen können.

HOLTZ und TRIEM (1937 b) fanden ferner, dass bei der Oxydation von Askorbinsäure, Cystein und Thioglykolsäure Wasserstoffsuperoxyd oder ein anderes Peroxyd entsteht, und sie sind der Meinung, dass die Desaminierung des Histidins durch Askorbinsäure dadurch ihre Erklärung finde.

Sowohl HOLTZ als EDLBACHER und v. SEGESSER (1937 c) weisen aber darauf hin, dass bei Einwirkung von Wasserstoffsuperoxyd

eine vollständige Desaminierung des Histidins unter Freiwerdung aller drei Stickstoffmoleküle erfolge, während bei Einwirkung der Askorbinsäure nur zwei frei gemacht werden.

Aus Anlass der vorstehend referierten Untersuchungen unternahmen GREENBLATT und PECKER (1940) einen Versuch, eine ähnliche Askorbinsäurewirkung in vivo nachzuweisen, indem sie untersuchten, ob man nach Injektion grösserer Askorbinsäuremengen (50 mg/Tag) bei Meerschweinchen und Kaninchen Veränderungen im Thionein- und Harnsäurespiegel des Blutes feststellen könne. Dies war indessen nicht der Fall.

In eigenen Versuchen (EKMAN 1941) konnte hingegen gezeigt werden, dass die Ausscheidung von Histidin im Harn bei Zufuhr von Askorbinsäure abnahm und bei Skorbut zunahm.

In Untersuchungen über den Umsatz von Thyrosin und Phenylalanin zeigten SEALOCK et al. (1939 a), dass bei Meerschweinchen nach Einverleibung dieser Aminosäuren ein gesteigerter C-Vitaminbedarf auftrat.

In fortgesetzten Untersuchungen fanden SEALOCK et al. (1939 b), dass Meerschweinchen mit Skorbutkost, wenn sie Thyrosin erhielten, »a substance of melanin-like properties» ausschieden, die sich bei der Analyse als Homogentisinsäure erwies. Aus Versuchen mit Meerschweinchen und Menschen ging hervor, dass die Ausscheidung von Homogentisinsäure (nach BRIGGS (1922) bestimmt) nach Einverleibung von Thyrosin ausblieb, wenn gleichzeitig Askorbinsäure verabfolgt wurde (5 mg/Tag und Meerschweinchen).

LEVINE et al. (1939) konnten im Harn von Neugeborenen mit mangelnder C-Vitaminzufuhr das Vorkommen von l—p-oxyphenylmilchsäure und wahrscheinlich von p-oxyphenylbrenztraubensäure nachweisen. Versuche, Thyrosin, Dihydroxyphenylalanin, Homogentisinsäure und Melanin nachzuweisen, missglückten.

Mittels der von FOLIN und CIOCALTEU (1927) angegebenen Methode zur Bestimmung von Monooxyphenylabkömmlingen konnte festgestellt werden, dass bei Zufuhr von 50—200 mg Askorbinsäure die Ausscheidung von Phenylderivat von 500—600 mg auf 40 mg absank, bei eingestellter Askorbinsäurezufuhr aber wieder anstieg.

In fortgesetzten Untersuchungen konnten SEALOCK et al. (1940 a) zeigen, dass Meerschweinchen, die eine askorbinsäurearme Kost und Thyrosin erhielten, nicht nur Homogentisinsäure ausschieden, sondern auch p-oxyphenylbrenztraubensäure, Oxyphenylmilchsäure und Thyrosin. In quantitativen Bestimmungen des »totalen Thyrosinwertes» nach FOLIN und CIOCALTEU (1927), der Ketosäure nach PENROSE und QUASTEL (1937) sowie der Homogentisinsäure nach BRIGGS (1922) konnte gezeigt werden, dass alle diese Thyrosinprodukte bei Zufuhr von 5—10 mg Askorbinsäure aus dem Harn verschwanden. Thyrosin wurde in Mengen von 500—1000 mg/Tag zugeführt, und die Verteilung auf die einzelnen Thyrosinmetaboliten in »per cent of theoretical calculated from amount of supplementary tyrosine eaten» war folgende: Ketosäure etwa 30 %, Thyrosin und Oxyphenylmilchsäure etwa 20 % und Homogentisinsäure 10—15 %. Ähnliche Resultate erhielten die Autoren bei Zufuhr von Phenylalanin. Wurde statt dessen d-Askorbinsäure einverleibt, so waren Tagesdosen von etwa 200 mg notwendig, um die Thyrosinderivate aus dem Harn verschwinden zu lassen. Dies ist 20—40mal so viel wie bei Verwendung von l-Askorbinsäure und steht in gutem Einklang mit der 20—50mal schwächeren antiskorbutischen Wirkung der d-Askorbinsäure.

In einer späteren Arbeit bestätigen SEALOCK et al. (1941) die Ergebnisse mit Phenylalanin und berichten über ähnliche Versuche mit Zufuhr von Phenylbrenztraubensäure und Oxyphenylbrenztraubensäure. Auch bei Einverleibung dieser Stoffe sahen sie bei Meerschweinchen mit Skorbutkost eine Ausscheidung derselben Metaboliten wie bei Zufuhr von Thyrosin und Phenylalanin. Die Produkte, die nach Einverleibung von Oxyphenylbrenztraubensäure ausgeschieden wurden, vor allem Ketosäuren, wurden indessen durch die Askorbinsäure so gut wie nicht beeinflusst.

Aus Anlass der vorstehend referierten Arbeiten untersuchten SEALOCK et al. (1940 b), ob es möglich sei, die Ausscheidung von Homogentisinsäure bei einem Alkaptonuriker zu beeinflussen. Dies misslang indessen, trotzdem sie bis zu 4 g Askorbinsäure pro Tag einverleibten.

2. Hormone.

a. *Thyroxin.*

Mosonyi (1933) hat gezeigt, dass die Gaswechselsteigerung, die man bei an Skorbut leidenden Meerschweinchen sieht, nach Einverleibung von Askorbinsäure verschwindet. Nach Kreitmair (1934) wird die gaswechselsteigernde Wirkung von Thyroxin auf Ratten geschwächt, wenn man gleichzeitig grosse Mengen (50 mg) Askorbinsäure verabfolgt.

Demole und Ippen (1935) haben gezeigt, dass Meerschweinchen mit beginnendem Skorbut durch Zufuhr von 0,5 mg Askorbinsäure geheilt werden können, dass die Tiere aber sterben, wenn sie gleichzeitig 0,1 mg Thyroxin bekommen. Steigert man nun die Askorbinsäuregabe auf 5 mg, so überleben einige Tiere, gibt man 10 mg, so überleben die meisten, und bei Gaben von 20 mg überleben alle. 20 mg Askorbinsäure sind dagegen wirkungslos, wenn die Thyroxingabe auf 0,2 mg erhöht wird. Diesen Versuch konnte Oehme (1936) bestätigen. Er vermochte zu zeigen, dass 0,1 mg Thyroxin keine Stoffwechselsteigerung bei Meerschweinchen bewirkt, wenn man gleichzeitig 20—25 mg Askorbinsäure verabfolgt. Ferner sahen auch Belasco und Murlin (1940) bei Ratten eine Schwächung der Grundumsatzsteigerung bei Zufuhr von 0,2 mg Thyroxin/100 g Körpergewicht, wenn sie gleichzeitig 20 mg Askorbinsäure/100 g Körpergewicht verabfolgten. In weiteren Untersuchungen zeigte Mosonyi (1935 und 1936), dass die Zufuhr von Thyroxin bei Meerschweinchen den Askorbinsäuregehalt der Nebennieren vermindert und dass in den Nebennieren von Ratten der Askorbinsäuregehalt ansteigt, wenn man die Schilddrüsen entfernt. Gleichsinnige Befunde erhoben Plaut und Bülow (1935) sowie Paal und Brecht (1937). Mosonyi (1936) stellte daraufhin die Theorie auf, dass mehrere Skorbutsymptome sich durch einen bei C-Vitaminmangel auftretenden Hyperthyreoidismus erklären liessen und dass ein Antagonismus zwischen Askorbinsäure und Thyroxin bestehe.

Diese Annahme wird ferner durch Aszodis (1937) Untersuchungen gestützt, aus denen hervorgeht, dass die bei Skorbut auftretenden Veränderungen des Blutbildes auch durch Verabfolgung von Thyroxin her-

vorgerufen werden können. Weiterhin lassen sich ALTENBURGERS (1936) Versuche hier heranziehen. Er zeigte, dass die Glykogenverarmung, die in der Meerschweinchenleber bei Zufuhr von 0,2 mg Thyroxin auftritt, durch Zufuhr von 10 mg Askorbinsäure verhindert werden kann. v. PLEHWE (1938) schliesslich zeigte, dass die bei Hyperthyreoidismus auftretende Kreatinurie verschwindet, wenn man Vitamin C darreicht.

Die Versuche, eine C-Vitamintherapie bei der BASEDOWschen Krankheit klinisch auszuwerten, schlugen indessen fehl (STRIECK 1935 und LÖHR 1936 konnten z. B. den Grundumsatz nicht durch Askorbinsäuregaben beeinflussen), und dass Askorbinsäure nicht immer antagonistisch gegenüber Thyroxin auftreten kann, zeigten SCHÄFERS (1936) Untersuchungen: im Kaulquappenversuch fehlte jede antithyreoïdale Wirkung. Schliesslich sei erwähnt, dass nach BAUCKE (1938) grosse Schwankungen des O_2-Verbrauchs (sowohl über als unter der Norm) beim Meerschweinchenskorbut karakteristisch ist.

b. *Adrenalin.*

Wie schon erwähnt, gab SZENT-GYÖRGYI (1930) an, dass Askorbinsäure die Oxydation von Polyphenolen, darunter auch die des Adrenalins, verhindern könne. ABDERHALDEN (1934) zeigte, dass die bei der Oxydation von Adrenalin oder Dioxyphenylalanin auftretende Farbstoffbildung bei Anwesenheit von Askorbinsäure nicht zustande kommt. Diese Versuche sowie der Nachweis sehr hoher Askorbinsäurekonzentrationen in den Nebennieren, dem Organ, wo das Adrenalin gebildet wird, gaben den Anlass, dass man die Askorbinsäure mit der Entstehung und Funktion des Adrenalins in Zusammenhang setzte.

Die Auffassung, dass das Adrenalin in vitro durch Askorbinsäure stabilisiert wird, haben mehrere Untersuchungen bestätigt (z. B. DAUOD und EL AYYADI 1938, MARQUARDT 1941). Nach GREEN und RICHTER (1937) verhindert Askorbinsäure die Oxydation über Orthochinon zu Adrenochrom.

SZENT-GYÖRGYIS Anschauung wird bestätigt durch WEBERS (1939) Untersuchungen, aus denen hervorgeht, dass Adrenalin, Corbasil, Sympathol, Veritol und Ephedrin ein und dieselbe Wirkung auf einen isolierten Kaninchendünndarm ausüben (Tonusabfall und Abnahme der Kontraktionshöhe). Diese Wirkung wird, was Adrenalin und Corbasil, beide mit zwei OH-Gruppen im Kern, betrifft, durch Askorbinsäure verstärkt und verlängert. Beim Sympathol, das nur eine OH-Gruppe hat, ist die Askorbinsäurewirkung gering, bei Veritol und Ephedrin, die keine OH-Gruppen im Kern aufweisen, ist die Askorbinsäure wirkungslos.

Nach v. EULER und KLUSSMANN (1933) sinkt der Adrenalingehalt der Nebennieren beim Meerschweinchenskorbut ab, und DOBY und WEISINGER (1938) kamen zu demselben Ergebnis. Umgekehrt konnte SARFY (1938) zeigen, dass bei Askorbinsäurezufuhr der Adrenalingehalt der Nebennieren und des Blutes anstieg. TONUTTI (1937) findet, dass die Askorbinsäure in den Nebennieren, die gewöhnlich in der Rinde lokalisiert ist, auch im Mark auftritt, wenn die Nebennieren zur Adrenalinproduktion angereizt werden. MOLNAR und FRIDRICH (1941) einverleibten einer grösseren Anzahl Versuchspersonen täglich 500 mg Askorbinsäure und konnten dann eine erhöhte Empfindlichkeit gegen die Injektion kleiner Adrenalingaben nachweisen.

Angesichts dieser sowie der auf S. 26 referierten Untersuchungen SEALOCKS und seiner Mitarbeiter erscheint ROTHMANS (1940) Theorie von der Rolle der Askorbinsäure in den Nebennieren bestechend. Nach seiner Anschauung ist die Aufgabe der Askorbinsäure »1) formation of cathecol precursors of epinephrine from phenol compounds of the proteins (phenylalanin, thyrosine) and 2) stabilisation of the epinephrine after it is formed in the suprarenal glands».

Indessen lassen sich gewichtige Gründe auch gegen diese Theorie anführen.

DOBY und WEISINGER (1938) fanden trotz der verminderten Adrenalinmenge in den Nebennieren bei Skorbut einen beträchtlich gesteigerten Adrenalingehalt des Blutes, und nach GIROUD und MARTINET (1941) findet man, wenn man die Adrenalinmenge pro Gewichteinheit des Nebennierenmarks berechnet, dass der Adrenalingehalt bei Skorbut ansteigt. In früheren Arbeiten ist der Adrenalingehalt für die ganze Nebenniere berechnet worden, und da sich das Mark bei Skorbut nicht ändert, die Rinde aber eine starke Hypertrophie erfährt, lassen sich so die Angaben betreffs eines verminderten prozentualen Adrenalingehaltes erklären.

Schliesslich kann man nach DAOUD und EL AYYADI (1938) in Meerschweinchenversuchen keinerlei Einfluss der Askorbinsäure auf die Adrenalinwirkung feststellen.

c. *Sexualhormone.*

MOSONYI (1936) hat gezeigt, dass wenn Meerschweinchenmännchen männliches Sexualhormon erhalten (Erugon, Igefa,

sowie Extrakt aus Stierhoden in Mengen $^1/_2$—1 Hahnenkamm-Einheit entsprechend), der C-Vitamingehalt der Leber und Nebennieren um 30—40 % abnimmt. Bei Weibchen rufen diese Präparate keine Wirkung hervor. Gibt man weiblichen oder männlichen Meerschweinchen Follikelhormon (5000 I. E. Progynon B. Schering oder 10000 M. E. Unden, Igefa), so ist in beiden Fällen eine Abnahme des C-Vitamingehaltes in Leber und Nebennieren nachzuweisen.

In einer folgenden Arbeit konnte Mosonyi (1937) zeigen, dass die Senkung des C-Vitamingehaltes durch Follikulin auch bei thyreoidektomierten wie kastrierten Männchen erzielt werden konnte, weshalb er der Ansicht ist, dass die Abnahme des C-Vitamingehaltes die Folge einer direkten Wirkung des Follikulins sein muss, das irgendwie antagonistisch zu Askorbinsäure steht.

Einen weiteren Beleg für diese Anschauung brachten die Untersuchungen von Sas (1936), in denen gezeigt wurde, dass der C-Vitamingehalt der Nebennieren männlicher Ratten nach Kastration anstieg. Hermann (1937) hat gefunden, dass sowohl die Zufuhr von Follikelhormon als von Askorbinsäure (50 mg) eine starke Steigerung des Glykogengehalts der Meerschweinchenleber bewirkt, während bei gleichzeitiger Verabfolgung dieser beiden Stoffe der Glykogengehalt der Leber abnimmt.

Schliesslich konnte Gergely (1938) an Meerschweinchenweibchen den Nachweis führen, dass die gasstoffwechselsteigernde Wirkung des Follikelhormons durch Darreichung von Askorbinsäure (50 mg) gehemmt werden kann.

Den Zusammenhang zwischen Sexualhormonen und Vitamin C beleuchten auch Pillays (1940) Untersuchungen, aus denen hervorgeht, dass die Askorbinsäureausscheidung im weiblichen Harn absinkt, wenn die Ovolution eintritt.

In eigenen Untersuchungen (Ekman 1941) ist gezeigt worden, dass die Desaminierung von Histidin durch Askorbinsäure in vitro durch einen Zusatz von Prolan gehemmt wird.

3. Toxine.

In der Literatur finden sich einige Angaben über die Entgiftung gewisser Toxine durch Askorbinsäure. Es handelt sich hier also um eine Askorbinsäurewirkung auf komplizierte Stoffe, deren Zusammensetzung in der Hauptsache unbekannt ist. Nach

den zur Verfügung stehenden Angaben scheint man indessen annehmen zu dürfen, dass das Toxinmolekül zyklische Komponenten enthält, z. B. zyklische Aminosäuren und auch andere zyklische Verbindungen.

Polonyi (1935) fand, dass mit Diphtherie infizierte Meerschweinchen niedrigere Askorbinsäurewerte in Leber und Nebennieren zeigten, als normale Tiere. Bekamen diese Tiere Askorbinsäure, so wuchs ihre Widerstandskraft gegen die Infektion. Mischte man Diphtherietoxin und Askorbinsäure und liess das Gemisch einige Zeit stehen, so wurde das Toxin entgiftet.

Greenwald und Harde (1935) sowie Jungeblut und Zwemer (1935) fanden, dass Diphtherietoxin, das man mit neutralisierter Askorbinsäure stehenliess, entgiftet wurde, und ferner, dass Meerschweinchen, denen eine sonst tödliche Diphtherietoxindosis einverleibt wurde, grössere Widerstandskraft zeigten, wenn sie grössere Mengen Askorbinsäure erhielten. Jungeblut und Zwemer sahen die stärkste entgiftende Wirkung in vitro bei einem Zusatz von 0,5—5 mg Askorbinsäure zu 2 Dos. Min. Leth. des Toxins, während sowohl grössere als kleinere Dosen weniger wirksam waren.

Die hier referierten Untersuchungen wurden von mehreren Seiten bestätigt, und man war recht allgemein der Anschauung, Askorbinsäure übe eine spezifische Wirkung auf Diphtherie aus, und die Befunde in vivo seien nicht nur auf die Behebung eines etwaigen Mangelzustandes zurückzuführen. Zilva (1937) wendet sich indessen gegen diese Anschauung und zeigt in einer grösseren Zahl von Versuchen, dass Vitamin C die Widerstandskraft von Meerschweinchen gegen Diphtherietoxin nicht zu steigern vermag.

Trotzdem die früheren Autoren behaupten, mit einer neutralisierten Askorbinsäurelösung gearbeitet zu haben, meinen Sigal und King (1937), dass die in vitro beobachtete Wirkung auf einer aspezifischen Säurewirkung des Vitamins C beruht habe, und sie geben an, dass sie bei einem pH des Gemischs von 6,4—7,4 keine Entgiftung haben feststellen können. Diese Autoren sind indessen der Meinung, dass die Voraussetzung für eine Entgiftung durch Askorbinsäure in den reduzierenden Eigenschaften dieser Säure zu suchen sei und heben hervor, dass bei ihren Versuchsbedingungen keine Oxydation der Askorbinsäure beobachtet werden konnte.

Versuche von entsprechender Art wie die hier betreffs des Diphtherietoxins angeführten sind auch mit anderen Toxinen gemacht worden. Nitzescu und Stan-Suciu (1940) zeigten, dass Kabragift, das man zusammen mit Askorbinsäure stehenlässt, entgiftet wird, und dass Meerschweinchen, denen kleine Mengen Kobragift durch Injektion einverleibt werden, einen sinkenden C-Vitamingehalt der inneren Organe zeigen.

4. Verschiedene zyklische Verbindungen von pharmakologischem oder toxikologischem Interesse.

A. Umwandlung und Entgiftung.

In mehreren Fällen hat man in vitro eine Umwandlung zyklischer Verbindungen durch Askorbinsäure nachweisen können und für einige dieser Verbindungen auch eine entsprechende Entgiftung in vivo.

Leibowitz und Guggenheim (1938) haben gezeigt, dass wenn Phenol in wässriger Lösung mit einer äquimolaren Menge Askorbinsäure versetzt wird, die beiden Stoffe miteinander reagieren. Diese Reaktion äussert sich darin, dass die Oxydationsgeschwindigkeit der Askorbinsäure zunimmt und sich die Gefrierpunktserniedrigung der Lösung verändert. Sie konnte auch dadurch nachgewiesen werden, dass das Gemisch Askorbinsäure/Phenol bei Injektion an Ratten weniger giftig war als Phenol allein.

Auf Grund dieser Befunde nehmen die Autoren an, dass das Phenol in gleicher Weise mit der Askorbinsäure gepaart worden ist, wie eine Koppelung an Glykuronsäure erfolgen kann. Und sie meinen, dass dem Versuch Bedeutung für das Verständnis der Funktion des Vitamins C zukommen: »Scurvy could very well be comprehended as an intoxication of the Vitamin C deficient organism with endogenously produced toxic substances of the normal metabolism which in the Vitamin C saturated organism are bound and detoxicated by ascorbic acid.»

Um weitere Beweise für diese Annahme zu erhalten, gaben sie Ratten eine vorbereitende Injektion von Askorbinsäure und anschliessend eine gleichmolare Menge Phenol. Indessen konnten sie unter diesen Umständen keine Entgiftung des Phenols feststellen.

Beyer (1941) fand, dass α-phenylisopropylamin (Amphetamin, Benzedrin) bei derselben Versuchsanordnung, wie sie Edlbacher und v. Segesser (1937) bei ihren Histidinversuchen angewandt hatten, durch Askorbinsäure desaminiert wird. Das Ergebnis konnte auch in vivo bestätigt werden: Hunde bekamen täglich eine gewisse Menge Benzedrin und die Ausscheidung im Harn wurde quantitativ bestimmt. Wurden ausserdem täglich 200—

400 mg Askorbinsäure verabfolgt, so sank die Benzedrinausscheidung auf etwa ein Drittel der Kontrollwerte.

In eigenen Versuchen (EKMAN 1942) ist durch quantitative Bestimmungen der zyklischen Verbindungen gezeigt worden, dass Phenol, Salizylsäure, Sulfanilamid, Sulfapyridin, Histidin, Indol und Tryptophan eine Umwandlung durch Askorbinsäure erleiden. Durch Versuche mit Benzol konnte wahrscheinlich gemacht werden, dass auch dieser Stoff eine ähnliche Umwandlung erfuhr. Die Versuchsanordnung war dieselbe wie die von EDLBACHER und v. SEGESSER (1937), und da die Bestimmungen von Phenol, Salizylsäure und Sulfanilamid auch nach Hydrolyse der Probelösungen dasselbe Resultat gaben, durfte angenommen werden, dass die Umwandlung nicht in einer Koppelung an die Askorbinsäure bestanden hatte. In Versuchen mit Meerschweinchen und Pflanzen (Kresse) konnte gezeigt werden, dass Phenol auch in vivo entgiftet wurde.

Bei der Verwendung von Benzol in verschiedenen Industrien haben sich Benzolvergiftungen als ein schwieriges berufshygienisches Problem erwiesen. MEYER (1937) weist auf eine gewisse Parallelität zwischen den Symptomen bei Skorbut und bei Benzolvergiftung hin und hat dementsprechend versucht, bei Meerschweinchen die Widerstandskraft gegen Benzol durch Darreichung von Askorbinsäure zu erhöhen.

Die Tiere bekamen 4mal wöchentlich 0,35 ccm Benzol subkutan einverleibt, und bei einer Tagesgabe von 20 mg Askorbinsäure liess sich ein deutlicher entgiftender Effekt feststellen. Das Versuchsmaterial war indessen nur klein. BORMANN (1937) und HAGEN (1937/38) machten in Kaninchenversuchen ähnliche Beobachtungen. LIBOWITZKY und SEYFRIED (1940) berichten, dass eine grössere Anzahl untersuchter Arbeiter, die der Einwirkung von Benzol ausgesetzt waren, Vergiftungsanzeichen namentlich im Frühjahr aufwiesen, und dass sämtliche in Belastungsversuchen dann einen verhältnismässig grossen C-Vitaminmangel an den Tag legten. Bei zusätzlichen C-Vitamingaben nahmen die Vergiftungserscheinungen bei gleicher Benzolexposition ab.

Schliesslich sei JORISSENS (1937) Beobachtung erwähnt, dass Naphtalin in vitro durch Askorbinsäure oxydiert werden kann.

Verschiedene Arzneien zyklischer Natur haben sich als weniger giftig erwiesen, wenn sie zusammen mit Askorbinsäure genommen werden.

Nach v. Auler (1935) wird die Toxizität von Germanin durch Askorbinsäure bei gleichbleibender Heilwirkung herabgesetzt.

Murakami (1939) und Onoyama (1939) haben gezeigt, dass die Fähigkeit der Leber, Santonin zu entgiften, im Verhältnis zum C-Vitamingehalt steht, und dass auch das lebende Versuchstier widerstandsfähiger gegen Santoninvergiftung ist, wenn es Askorbinsäure erhält.

Borsetti (1938) fand, dass Meerschweinchen, die täglich 50—100 mg Askorbinsäure bekommen, eine sonst tödliche Atophandosis (Phenylchinolinkarbonsäure) überleben, und Tagariello (1939) hat gezeigt, dass Hunde, die täglich 300 mg Askorbinsäure bekommen, trotz Tagesgaben von 2 g Atophan keine degenerativen Veränderungen der inneren Organe aufweisen.

Haas (1939) sah, dass in Ratten-, Meerschweinchen- und Kaninchenversuchen die bei den Kontrolltieren auftretende Temperatursteigerung ausblieb, wenn die Versuchstiere 25 mg Dinitrophenol pro kg Körpergewicht, gleichzeitig aber auch mindestens 250 mg Askorbinsäure pro kg Körpergewicht erhielten.

Nach Büssing (1939) stellen sich bei Kaninchen akute Vergiftungserscheinungen ein, wenn die Tiere 30 mg Chinin bekommen. Injiziert man aber gleichzeitig 100 mg Askorbinsäure, so bleiben diese Vergiftungserscheinungen aus.

Rosenthal (1939) fand in Meerschweinchenversuchen, dass die Toxizität von Sulfanilamid abnahm, wenn die Tiere mit an Vitamin C reichen Vegetabilien (Kohl) gefüttert wurden, und nach Dainow (1941) werden die Nebenwirkungen von Sulfonamidpräparaten gemildert, wenn man gleichzeitig Askorbinsäure verabfolgt.

Seiferth und Kolb (1939) konnten durch Tagesgaben von 500 mg Askorbinsäure einer Vergiftung bei langdauernder Zufuhr von toxischen Dosen (etwa 5 mg/Tag) AT 10 (Dihydrotachysterin) vorbeugen. Als Versuchstiere hatten sie Kaninchen.

Im Schrifttum finden sich zahlreiche Angaben über mildernde Wirkung der Askorbinsäure auf toxische Nebenerscheinungen bei Salvarsanbehandlung. Hier wäre jedoch teils eine stabili-

sierende Wirkung der Askorbinsäure auf die Arsenikkomponente, teils auch eine Umwandlung der zyklischen Komponente denkbar. Die Anregung, Salvarsanbehandlung mit erhöhter Askorbinsäurezufuhr zu verbinden, scheint von DAINOW (1937) zu stammen. Er fand, dass die Giftwirkung des Salvarsans schwächer ist, wenn man eine Salvarsanlösung mit Askorbinsäure versetzt.

Einen ähnlichen Versuch machte DUREL (1937). Er spritzte Mäusen intravenös eine sicher tödliche Neosalvarsandosis ein, zugleich aber gruppenweise verschiedene Askorbinsäuregaben. Je grösser die verabfolgte Askorbinsäuremenge, um so grösser auch die Zahl der überlebenden Mäuse. In einem anderen Versuch wurde Mäusen eine Naganainfektion beigebracht. Die Tiere erhielten dann eine gut heilend wirkende Neosalvarsandosis, wie im vorigen Versuch aber mit verschieden grossen Askorbinsäuregaben. Es zeigte sich, dass die heilende Wirkung um so geringer war, je mehr Askorbinsäure zugesetzt wurde.

DAINOW (1937) untersuchte die C-Vitaminsättigung bei verschiedenen luischen Patienten und konnte zeigen, dass diejenigen, die Überempfindlichkeit gegen Salvarsan zeigten, an starkem C-Vitaminmangel litten. Ähnliche Befunde erhob KÜHNER (1941). Diese Erfahrungen lassen sich mit dem von FASAL (1933) erhobenen Befund in Zusammenhang bringen, dass Salvarsanüberempfindlichkeit namentlich im Winter und Frühjahr, der Zeit des geringsten C-Vitamingehaltes der Nahrung, auftritt. (Dieselbe Erfahrung meldet WELCKER [1940].) DAINOW (1937) gab infolgedessen Einspritzungen von Askorbinsäure und Neosalvarsan im Verhältnis 1 : 10 und fand eine gleichbleibende Heilwirkung des Salvarsans, während die toxischen Nebenwirkungen ausblieben. Er sucht die Wirkung der Askorbinsäure in der Fähigkeit, das Salvarsan zu stabilisieren und eine zu schnelle Oxydation der Arsenikkomponente zu verhindern.

Gegen das DAINOWsche Verfahren richtet sich indessen WELCKER (1940), der angibt, er könne keinerlei Zusammenhang zwischen C-Vitaminsättigung und Salvarsanüberempfindlichkeit feststellen; ferner würden mit Askorbinsäure versetzte Salvarsanlösungen schnell dekomponiert.

B. Die Einwirkung zyklischer Verbindungen auf den Vitamin-C-Umsatz.

Im vorigen Abschnitt wurden zahlreiche Arbeiten erwähnt, die von Entgiftung und Umwandlung zyklischer Verbindungen durch Askorbinsäure berichten. Diese Befunde haben sich in vielen Fällen durch abnehmende Askorbinsäurewerte im Serum, Harn oder inneren Organen bei Zufuhr zyklischer Verbindungen im Meerschweinchen- oder Menschenversuch bestätigen lassen.

So fand FRIEMANN (1936) bei benzolexponierten Arbeitern eine geringere Askorbinsäureausscheidung im Harn als bei anderen Menschen. BORMANN (1937) und HAGEN (1937/38) fanden entsprechend bei benzolexponierten Arbeitern niedrigere Serumwerte. POUMEAU-DELILLE (1941) sah bei Meerschweinchen nach Einverleibung von Benzol einen niedrigeren Askorbinsäuregehalt in den Nebennieren, während der Gehalt der Leber normal war.

Nach DAINOW und ZIMMET (1939) sinkt der Askorbinsäuregehalt der Leber, Nebennieren, Hoden und des Gehirns bei Meerschweinchen, die gewisse Sulfonamidpräparate erhalten.

FARRER et al. (1940) konnten zeigen, dass der Askorbinsäuregehalt des Plasmas bei Lueskranken abnahm, wenn diese mit Neosalvarsan behandelt wurden.

Einen gerade entgegengesetzten Befund, nämlich vermehrte Ausscheidung von Askorbinsäure im Harn, erhoben dagegen DANIELS und EVERSON (1936) bei Kindern, die Azetylsalizylsäure bekamen. Die normale Ausscheidung von 25—35 mg pro Tag verdoppelte sich. Die Askorbinsäure wurde nach BIRCH, HARRIS und RAY (1933) bestimmt. SAMUELS et al. (1940) konnten die Befunde in Meerschweinchen- und Rattenversuchen bestätigen.

Bei Tieren aber, die selbst Vitamin C synthetisieren, z. B. Ratten, scheint es, als wäre eine vermehrte Erzeugung von Askorbinsäure bei Zufuhr zyklischer Verbindungen eine natürliche Abwehrreaktion gegen die vergiftenden Wirkungen.

LONGENECKER et al. (1940) untersuchten die Ausscheidung von Askorbinsäure im Harn von Ratten, die eine Standardkost bekamen, zusätzlich aber 20 mg verschiedener Pharmaka pro Tag, die ursprünglich unter den Stoffen »functioning as nerve depressants» gewählt worden waren.

Von 11 untersuchten Barbitursäurederivaten erwies sich das »Phenobarbital» (Äthylphenylbarbitursäure) am wirksamsten und verursachte eine Steigerung der ursprünglichen Askorbinsäureausscheidung von 0,2 mg pro Tag auf etwa 10 mg. Zur Kontrolle der Vitamin-C-Bestimmungsmethodik (BIRCH, HARRIS, RAY 1933) wurde eine biologische Nachprüfung vorgenommen, die dasselbe Resultat lieferte. Die schwächste Wirkung hatte »Amytal» (Isoamylbarbitursäure), welcher Stoff die Askorbinsäureausscheidung nur auf etwa 1 mg pro Tag steigerte.

Von den mit anderen zyklischen Verbindungen gleichartiger Wirkung gewonnenen Resultaten sei erwähnt, dass Aminopyrin und Antipyrin eine Steigerung auf etwa 6 mg bewirkten, während die Askorbinsäureausscheidung nach Zufuhr von Azetanilid und Phenazetin nur 0,5—1,3 mg pro Tag war.

Auch nicht-zyklische Hypnotika wurden geprüft. Paraldehyd bewirkte eine ebenso starke Steigerung wie Phenobarbital, Chloreton gar eine noch stärkere. Auch andere zyklische Verbindungen wurden indessen untersucht, und von diesen bewirkten z. B. Sulfanilamid, Sulfapyridin und Phenol eine Steigerung der Askorbinsäureausscheidung im Harn auf 0,5—1,5 mg pro Tag.

Die Autoren meinen zwar, die gesteigerte Askorbinsäureproduktion nach Einverleibung der untersuchten Stoffe könne zum Ziele einer Entgiftung erfolgt sein. Gegen diese Annahme führen sie aber an, dass eine Koppelung der einverleibten Verbindungen an die ausgeschiedene Askorbinsäure, beispielsweise analog der Koppelung an Glykuronsäure, nicht nachgewiesen werden konnte.

Svirbely (1939) untersuchte an Ratten die Reduktionsfähigkeit gegenüber Dichlorphenolindophenol in Leber und Nebennieren und fand bei Tagesgaben von 25—50 mg Kupfersulfat keine Veränderungen. Bei Darreichung von Pyridin, Menthol, Borneol, Thymol, Kampfer, Phenylessigsäure, Amidopyrin und Antipyrin stieg indessen die Reduktionsfähigkeit in Leber und Darm. Nach Zufuhr von Azetanilid, Azetophenetidin, Amidopyrin, Kampfer, Borneol, Toluylendiamin und Hydrazin nahm der Askorbinsäuregehalt der Nebennieren ab. Die Menge der verabfolgten Verbindungen belief sich im allgemeinen auf Tagesgaben von 100 mg und mehr an 3—5 Tagen vor der Untersuchung. (Die Toluylendiamingabe war 32 mg, die Hydrazingabe 15 mg.)

Zusammenfassung und Besprechung der einschlägigen Literatur.

Die Beziehungen zwischen Vitamin C, Farbstoffbildung und zyklischen Verbindungen lassen sich in folgenden Hauptpunkten zusammenfassen:

I. *Umwandlung zyklischer Verbindungen durch Askorbinsäure in vitro.*

Die Umwandlung entsprach

einer Desaminierung (Phenylalanin, Thyrosin, Tryptophan, Histidin, α-phenylisopropylamin. Im Falle des Histidins war eine Ringspaltung nachzuweisen.),

einer Oxydation (Naphtalin),

(beim Phenol vermutlich) einer Koppelungsreaktion.

Der chemische Hergang war unbekannt, liess sich aber dadurch nachweisen,

dass die Konzentration der zyklischen Verbindung nach beendigtem in-vitro-Versuch niedriger war (Histidin, Tryptophan, Indol, Phenol, Salizylsäure, Sulfanilamid und Sulfapyridin),

dass die Toxizität einer bestimmten Menge der unbehandelten zyklischen Verbindung grösser war als die einer gleich grossen Menge der Verbindung nach deren Reaktion mit Askorbinsäure (Diphtherietoxin, Kobratoxin, Phenol, Salvarsan).

II. *Umwandlung zyklischer Verbindungen durch Askorbinsäure in vivo.*

Diese wurde dadurch nachgewiesen,

dass die Ausscheidung der zyklischen Verbindung im

Harn durch Verabfolgung von Askorbinsäure vermindert werden konnte (Histidin, Thyrosin, Phenylalanin, Phenylbrenztraubensäure, Phenol, α-phenylisopropylamin),
dass die zyklische Verbindung weniger giftig war, wenn gleichzeitig Askorbinsäure einverleibt wurde (Thyroxin, Benzol, Sulfonamidpräparate, Germanin, Atophan, Chinin, Santonin, Dihydrotachysterin, Salvarsan).

III. *Eine antagonistische Wirkung der Askorbinsäure in vivo*

(gegenüber Thyroxin, gewissen Sexualhormonen, Dinitrophenol).

IV. *Stabilisierung leicht oxydabler zyklischer Verbindungen durch Vitamin C in vivo und in vitro.*

(Adrenalin, [Salvarsan], Vorstufen der Harnfarbstoffe [Urochrom A] und der Pigmente.)

V. *Einwirkung zyklischer Verbindungen auf den C-Vitaminumsatz in vivo.*

Diese konnte nachgewiesen werden durch
vermehrte Ausscheidung von Askorbinsäure im Harn bei der Ratte nach Einverleibung von Barbitursäure, Sulfanilamid, Sulfapyridin, Phenol, Salizylsäure, Aminopyrin, Antipyrin, Azetanilid, Phenazetin,
verminderten Askorbinsäuregehalt der Gewebe bei der Ratte oder beim Meerschweinchen nach Einverleibung von Toluylendiamin, Azetanilid, Azetophenetidin, Amidopyrin, Kampfer, Borneol, Hydrazin, Benzol, Sulfonamidpräparaten, Thyroxin, verschiedenen Sexualhormonen,
grösseres Defizit bei Belastungsversuchen am Menschen (Benzol), Senkung des Askorbinsäuregehaltes des Plasmas (Neosalvarsan, Benzol).

Es liegen also zahlreiche Beispiele von Reaktionen zwischen Vitamin C und zyklischen Verbindungen in vitro wie in vivo vor.

In einem Falle hat man die Rolle der Askorbinsäure sowohl in vitro als in vivo erklären können, nämlich was die Stabilisierung leicht oxydabler zyklischer Verbindungen durch Askorbinsäure betrifft. Die Wirkung gründet sich hier auf die reduzierenden

Eigenschaften der Askorbinsäure und äussert sich in vitro z. B. darin, dass die Entstehung farbiger Oxydationsprodukte verhindert wird, in vivo z. B. als eine Verstärkung der Adrenalinwirkung und eine Abnahme der Farbstoffbildung und Pigmentierung. Auch betreffs der Entgiftung von Salvarsan hat man dieselbe Erklärung angenommen: die Wirkung der Askorbinsäure solle auf einer Hemmung der Arsinoxydbildung beruhen. Nach dem referierten Versuchsmaterial zu urteilen, kann es jedoch ebensogut so sein, dass das Salvarsan eine Umwandlung vom selben Schlage wie z. B. einfache aromatische Verbindungen (Phenol u. a.) erfährt.

Im übrigen dürfte indessen der Verlauf in vivo ganz unbekannt sein. Als Erklärung der beobachteten Umwandlung von Aminosäuren in vitro hat man einen oxydativen Vorgang angenommen. Da sich die Entstehung von Wasserstoffsuperoxyd oder anderen Peroxyden bei Oxydation von Askorbinsäure hat nachweisen lassen, ist die Möglichkeit erörtert worden, dass die Oxydation durch die besagten Stoffe herbeigeführt worden sei.

Das Vitamin-C-Schrifttum ist hier zwar nur soweit referiert worden, als es auf den Zusammenhang von zyklischen Verbindungen und Askorbinsäure Bezug hat. Trotzdem dürften die angezogenen Arbeiten grosse Teile der gegenwärtigen Einsicht in die chemische und pharmakologische Funktion des Vitamins C umfassen. Soweit ich habe sehen können, findet sich indessen in der Literatur kein Hinweis darauf, dass das Vitamin C von spezieller Bedeutung für den Umsatz gerade zyklischer Verbindungen wäre.

Dies dürfte folgende Gründe haben:

In sehr hohem Grade war man darauf eingestellt, dass die hervorstechendste chemische Eigenschaft der Askorbinsäure ihr Reduktionsvermögen sei und dass dieses weitgehend auch ihre physiologische Bedeutung bedinge. Wie schon erwähnt, vermag dieses auch gewisse der beobachteten Reaktionen zu erklären. Bei den übrigen reicht es indessen nicht aus, und die Theorien über die Bedeutung des Auftretens von Peroxyden bei der Oxydation der Askorbinsäure haben keine Beachtung gefunden. Was zyklische Verbindungen und Vitamin C betrifft, erscheint es

indessen wesentlich, festzuhalten, dass Vitamin C sowohl umwandelnd als stabilisierend wirken kann.

Bei den Versuchen, Entgiftung durch Askorbinsäure nachzuweisen, ist man im allgemeinen von der Voraussetzung ausgegangen, die Entgiftung vollziehe sich durch eine Koppelung der Askorbinsäure und der zyklischen Verbindung. Da eine solche Koppelung in keinem Falle hat nachgewiesen werden können, hat man sich zu den gewonnenen Resultaten ablehnend verhalten.

Auch andere Verbindungen als zyklische haben in vitro durch Askorbinsäure umgewandelt werden können. Angaben über eine ähnliche Einwirkung in vivo dürften jedoch selten sein.

Der Antagonismus zwischen Vitamin C und Thyroxin sowie gewissen Sexualhormonen hat ziemliches Interesse erweckt. Man hat indessen diesen Antagonismus als eine Folge davon aufgefasst, dass das Vitamin und die Hormone gewisse Reaktionen im Organismus in entgegengesetztem Sinne beeinflussten. Ebenso wahrscheinlich dürfte es aber sein, dass in einem akuten Versuch mit gleichzeitiger Einverleibung von grossen Mengen Vitamin und Hormon das Hormon direkt durch das Vitamin umgewandelt wird. Dies wäre eine direkte Analogie zur Stabilisierung des polyphenolähnlichen Adrenalins durch Vitamin C unter den entsprechenden Bedingungen, doch ist in dem Falle selbstverständlich ein Synergismus die Folge. In den Versuchen, in denen man einen gesteigerten C-Vitaminverbrauch nach Zuführung von Hormonen gefunden hat, dürfte die Erklärung — in Anbetracht der kleinen Hormonmengen — am ehesten darin zu suchen sein, dass die fraglichen Hormone durch ihre stoffwechselsteigernde Wirkung indirekt den C-Vitaminbedarf ebenfalls steigern.

Fragestellung.

Eigene frühere Untersuchungen sowie Angaben in der Literatur lassen erkennen, dass die Askorbinsäure für den Umsatz zyklischer Verbindungen und für die Bildung von Urochrom A wichtig ist.

Die vorliegende Untersuchung verfolgt die Absicht, den Zusammenhang zwischen Vitamin C, zyklischen Verbindungen

und Urochrom A näher zu beleuchten; folgende Probleme stehen zur Behandlung:

Welche Faktoren können auf die Umwandlung zyklischer Verbindungen durch Askorbinsäure unter Bildung von Urochrom A in vitro einwirken?

Lässt sich eine Umwandlung zyklischer Verbindungen durch Askorbinsäure unter Bildung von Urochrom A auch in vivo nachweisen?

Welcher chemische Hergang liegt der Umwandlung der zyklischen Verbindung und der Bildung des Urochroms A zugrunde?

Ist das Urochrom A ein einheitlicher Stoff?

Kann die Umwandlung zyklischer Verbindungen als eine wesentliche Funktion des Vitamins C gelten?

Um diese Probleme zu untersuchen, muss man die reagierenden Stoffe (Askorbinsäure und zyklische Verbindung) und das Produkt (Urochrom A) bei Versuchen in vitro sowohl als in vivo quantitativ bestimmen. Man muss also solche zyklischen Verbindungen untersuchen, die wir quantitativ bestimmen können.

In früheren Untersuchungen (Ekman 1942) wurde gezeigt, dass der Histidinumsatz durch Vitamin C beeinflusst wird, ebenso ein Benzolderivat, das Phenol.

Da es sich nun darum handelte, die bei der Umwandlung zyklischer Verbindungen obwaltenden Verhältnisse näher zu untersuchen, erschien es wünschenswert, neue zyklische Verbindungen in den Rahmen der Untersuchung einzubeziehen. Die Wahl fiel auf Indol, Benzol, Sulfanilamid und Salizylsäure, und zwar aus folgenden Gründen:

1. Das Indol repräsentierte eine bisher nicht untersuchte Gruppe zyklischer Verbindungen.

2. Das Phenol ist ein Monooxybenzol, und durch die Wahl von Benzol, Salizylsäure und Sulfanilamid wurde erreicht, dass die Untersuchung teils die denkbar einfachste Benzolverbindung und teils kompliziertere Abkömmlinge umspannte: die Salizylsäure mit einer Karboxylgruppe und das Sulfanilamid mit einer Aminogruppe und einer Sulfonamidgruppe. Das Benzol konnte

zwar nicht quantitativ bestimmt werden, doch hatte diese Verbindung dank ihrer Flüchtigkeit und geringen Löslichkeit in Wasser wesentliche Vorzüge, wenn es galt, die Umwandlungsprodukte in den Versuchen in vitro zu untersuchen.

3. Weder der Salizylsäure-, Sulfanilamid- noch der Benzolumsatz können bei Versuchen in vivo dadurch beeinflusst werden, dass diese Stoffe in der Nahrung vorkommen, was bei den in früheren Arbeiten untersuchten Verbindungen (die Aminosäure Histidin und Phenol, das beim Umsatz gewisser Aminosäuren freigemacht werden kann) der Fall war.

4. Der Umsatz von Benzol und Indol sowie von Salizylsäure und Sulfanilamid ist an und für sich von Interesse, da diese Stoffe aktuelle toxische Verbindungen bzw. vielgebrauchte Heilmittel sind.

KAP. II.

Methodik.

I. Quantitative Bestimmung des Urochrom A.

In einer früheren Arbeit (Ekman 1940) wurde eine Methode zur quantitativen Bestimmung von Urochrom A angegeben, die sich auf die Ausfällbarkeit des Urochroms mit Kupferazetat gründet. Das Prinzip der Methode ist folgendes:

Man vergleicht die Farbe einer Lösung, die nach vorbereitender Ausfällung von Phosphaten und Sulfaten mit Kupferazetat gefällt und dann mit Salzsäure gekocht worden ist, mit der Farbe einer anderen Portion derselben Lösung, die zwecks Kompensation der Fällungslösungen mit Aq. dest. versetzt und dann ebenfalls mit Salzsäure gekocht worden ist. Der Farbunterschied ist ein Ausdruck für die Urochrom-A-Konzentration, und er lässt sich direkt ablesen, indem man mit der gefällten Lösung in der einen Küvette und der nicht gefällten als Vergleichslösung in der anderen kolorimetriert. (Dasselbe Prinzip beim Kolorimetrieren wurde von Grönwall (1938) in ihrer »kolorimetrischen Methode zur Bestimmung der Indolderivate im Harn» verwendet.)

Bei der Kolorimetrie enthält folglich die eine Portion Kupferazetat und die andere nicht. Es hat sich gezeigt, dass die Anwesenheit gewisser aromatischer Verbindungen, die bei Versuchen in vivo und in vitro zur Anwendung gelangen werden, in der Probelösung eine Farbreaktion mit Kupferazetat bewirkt, welche die Urochrom-A-Bestimmung stört. Um diese Fehlerquelle auszuschalten, muss man also darauf achten, dass beide Portionen gleich grosse Mengen Kupferazetat enthalten. Urochrom A lässt

sich mit Kupferazetat nur in neutraler oder schwach saurer Lösung fällen, während die Färbung der aromatischen Verbindungen mit Kupferazetat dieselbe ist, unabhängig davon, wann die Kupferazetatlösung zugesetzt wird. Bringt man also die Kupferazetatlösung erst nach dem Zusatz von Salzsäure in die Vergleichslösung, so wird die Urochrom-A-Konzentration nicht beeinflusst, während dagegen etwa vorhandene aromatische Verbindungen eine in beiden Lösungen gleich starke Färbung bewirken und somit die Urochrombestimmung nicht stören.

Die Urochrom-A-Bestimmung geschah in dieser modifizierten Form nach folgendem Arbeitsschema:

(Zum Kochen werden Reagensgläser von 50 ccm aus Jenaglas verwendet, die mit einem Merkstrich für 20 ccm und 2 Glasperlen versehen sind. Im übrigen werden gewöhnliche Reagensgläser benutzt.)

1. 20 ccm Probelösung werden mit 2 ccm Bariumazetatlösung (53 g—1000 ccm Aq. dest.) und 1 ccm 10 % Ammoniak versetzt. Schütteln, dann Erhitzen über dem Bunsenbrenner, bis sich ein grobflockiger Niederschlag von Phosphaten zeigt. Filtrieren.

2. 10 ccm des Filtrats von 1. werden mit 10 ccm 5 % Kupferazetatlösung versetzt. Das Gemisch wird über Nacht stehengelassen und am nächsten Tage filtriert.

3. a) In ein Kochreagensglas werden 5 ccm des Filtrats von 1., 10 ccm 1n HCl und 5 ccm 5 % Kupferazetatlösung gegeben.

b) In ein zweites werden 10 ccm des Filtrats von 2. und 10 ccm 1n HCl gebracht und 5 Min. auf einem Kjeldahl-Mikroofen gekocht. (In beide Reagenzgläser werden 2 Glasperlen gelegt, um Stossen zu verhindern.)

4. Nach dem Erkalten wird bis zur Marke verdünnt, filtriert und im Zeissschen Pulfrich-Stufen-Photometer kolorimetriert, wobei die beiden grössten Küvetten (30 mm) und das Filter S47 verwendet werden.

(Extinktionswerte über 1,4 sind der Urochrom-A-Konzentration nicht direkt proportional. Ergeben sich solche Werte, so muss die Bestimmung nach geeigneter Verdünnung der Probelösung wiederholt werden.)

Angabe der Ergebnisse.

Da bisher noch keine ausreichenden Mengen eines definierten Urochrom-A-Präparats haben dargestellt werden können, konnte die Bestimmungsmethode nicht kalibriert werden, sondern sie liefert nur relative Werte.

Damit diese vergleichbar werden, wird der auf dem Photo-

meter abgelesene Wert (der Extinktionswert = E) bei Bestimmung nach der besagten Methodik und bei einer bestimmten Harnmenge pro Tag angegeben. Bei Versuchen mit Meerschweinchen z. B. ist der im Verlauf von 24 Stunden gesammelte Harn auf 25 ccm verdünnt worden. Bisweilen betragen die Harntagesmengen über 25 ccm, da aber, wie aus den Verdünnungsversuchen, S. 51, für die angegebene Methodik das BEERsche Gesetz gilt, kann man diese Werte direkt auf die Tagesmenge 25 ccm umrechnen.

Bei Versuchen mit Tieren von erheblichen Gewichtsunterschieden ist die Urochrom-A-Ausscheidung als Index laut folgender Formel angegeben worden:

$$\text{Urochrom-A-Index} = \frac{E \times 10000}{g}$$

E ist der Extinktionswert bei Bestimmung in einer definierten Tagesmenge Harn, g das Gewicht des Tieres in Gramm (Multiplikation mit 10000, um Dezimalstellen zu vermeiden).

Prüfung der Methode.

Die Konzentrationen der angewandten Lösungen stimmen mit denen überein, die DOMBROWSKI (Seite 13) bei seinen Versuchen zur Isolierung von Urochrom verwendet hat. Die Lösungen, die für Urochrom-A-Bestimmungen in Frage kommen, Harn und Lösungen von Versuchen in vitro, können indessen in ihrer Zusammensetzung hinsichtlich anderer Stoffe höchst beträchtliche Variationen aufweisen. In Ermangelung eines zweckdienlichen Urochrom-A-Präparats lässt sich die Methodik nicht in gewohnter Weise durch Zusatz bekannter Urochrommengen zu verschiedenen »Probelösungen» kontrollieren. Man kann indessen feststellen, ob unterschiedliche Konzentrationen der Reagenslösungen grosse Veränderungen der abgelesenen Werte zur Folge haben. Bleiben die Werte stabil, so wird man erwarten dürfen, dass die Methode auch in unterschiedlichen Milieus reproduzierbare Werte liefern wird.

TABELLE 2.

Die Einwirkung von Änderungen der Bariumazetatkonzentration auf den Extinktionswert.

Bariumazetat g/l		26,5	53	106
Menschenharn	E	0,29	0,25	0,21
Meerschweinchenharn	E	0,91	0,76	0,86
»Histidinversuch»	E	1,13	0,93	0,71

TABELLE 3.

Die Einwirkung von Änderungen der Ammoniakkonzentration auf den Extinktionswert.

Ammoniaklösung %		5	10	15	20
Menschenharn	E	0,25	0,27	0,34	0,34
Meerschweinchenharn	E	0,85	0,86	0,79	0,74
»Histidinversuch»	E	0,97	1,02	1,02	1,00

TABELLE 4.

Die Einwirkung von Änderungen der Kupferazetatkonzentration auf den Extinktionswert.

Kupferazetatlösung %		4	5	6
Menschenharn	E	0,37	0,35	0,32
Meerschweinchenharn	E	0,74	0,81	0,82
»Histidinversuch»	E	1,06	0,95	0,87

TABELLE 5.

Die Einwirkung von Änderungen der Salzsäurekonzentration auf den Extinktionswert.

Salzsäure n		0,5	1	2
Menschenharn	E	0,26	0,25	0,265
Meerschweinchenharn	E	0,57	0,80	0,83
»Histidinversuch»	E	1,0	0,95	0,92

Wie nun aus den Tabellen 2—5 ersichtlich ist, bewirken auch sehr starke Schwankungen in der Zusammensetzung der Bariumazetat-, Ammoniak-, Kupferazetat- und Salzsäurelösungen nur geringfügige Schwankungen der abgelesenen Werte, einerlei ob die Probelösung menschlicher Harn, Meerschweinchenurin oder eine Lösung von einem Versuch in vitro ist. (In der Spalte »Histidinversuch» in den Tabellen handelt es sich um Lösungen, die bei Abbauversuchen mit Histidin durch Askorbinsäure erhalten worden sind.)

Tabelle 6 zeigt, dass die Fällung mit Kupferazetat bei schwächeren Urochrom-A-Konzentrationen schon nach 3 Stunden vollständig ist, bei hohen Konzentrationen nach 21 Stunden. Die Vorschrift des Arbeitsschemas, die Probelösungen an dem einen Tage zur Fällung anzusetzen und sie erst am nächsten Tage fertigzumachen, gewährleistet also wohl eine vollständige Fällung.

Wie aus Tabelle 7 hervorgeht, erhält man dasselbe Ergebnis bei Anwendung der ursprünglichen oder der modifizierten Methode, soweit es sich um normalen Harn handelt, während in salizylsäurehaltigem Harn das ursprüngliche Verfahren bedeutend niedrigere Werte gibt.

Nach den übereinstimmenden Angaben des einschlägigen Schrifttums ist das Urochrom eine leichtoxydable Verbindung, deren fortschreitende Oxydation wenigstens zum Teil der Grund für die sog. Nachdunkelung des Urins ist. Bei einem kolorimetrischen Verfahren zur Bestimmung von Urochrom A muss man deshalb zusehen, dass die Bestimmung mit Urochrom in einem definierten Oxydationsstadium erfolgt, und zwar am einfachsten nach vollständiger Oxydation. Aus diesem Grunde werden die einzelnen Harnportionen vor dem Kolorimetrieren gekocht.

Bei Behandlung mit Säuren in Wärme konnte Dombrowski (Seite 13) ein Melanin aus Urochrom erhalten, weshalb also die Kochzeit nicht zu lang gewählt sein darf. Wie Tabelle 8 zeigt, erhält man bei 4—6minütigem Kochen konstante Ableswerte und eine Kochzeit von 5 Min. erscheint also zweckmässig.

Wie aus Tabelle 9 ersichtlich, ist die Kolorimetrierfarbe wenigstens 24 Stunden lang haltbar, was von praktischer Bedeutung ist. Es besagt ausserdem, dass die durch das Kochen erzielte Oxydation des Urochroms vollständig war, da ein weiteres Nachdunkeln nicht eintritt.

Dieselben Befunde, die in den Tabellen 6—9 für Meerschweinchenharn angegeben sind, habe ich in ähnlichen Versuchen mit Menschen-Urin und mit aus Versuchen in vitro gewonnenen Lösungen erhoben.

Um zu untersuchen, ob direkte Proportionalität zwischen den Extinktionswerten und den Urochromkonzentrationen besteht, d. h. ob das Beersche Gesetz für die Methode gültig ist, wurde folgendermassen verfahren.

Relativ konzentrierte Urine oder Lösungen aus Versuchen in vitro wurden mit Aq. dest. aufgefüllt, so dass die Summe von Urochromlösung und Aq. dest. stets 20 ccm war, d. h. die Menge, mit der die Bestimmung

TABELLE 6.

Bestimmung der Zeit, die zur vollständigen Ausfällung mit Kupferazetat benötigt wird. (Meerschweinchenharn)

Stunden	Harn Nr.		
	1	2	3
3 E	0,37	0,98	0,94
8,5 E	0,37	0,93	0,97
21 E	0,37	1,18	1,18
30 E		1,13	1,21

TABELLE 7.

Vergleich zwischen den Extinktionswerten, nach der ursprünglichen und der modifizierten Methodik erhalten, bei normaler Kost und bei Verabreichung von Natriumsalizylat.

Meerschweinchen Nr.	E		
	Mit CuAc	Ohne CuAc	
1	0,58	0,61	Gewöhnliche Kost
2	0,50	0,52	
3	0,43	0,43	
4	0,54	0,44	Diese Tiere erhielten täglich Natriumsalizylat
5	0,95	0,48	
6	0,44	0,07	

TABELLE 8.

Bestimmung der Kochzeit. (Meerschweinchenharn)

Kochzeit in Minuten:	3	4	5	6	8
E (Mittl. Wert)	0,47	0,463	0,463	0,46	0,445
Anzahl d. Bestimmungen	1	3	3	2	2

TABELLE 9.

Haltbarkeit der Kolorimetriefarbe. (Meerschweinchenharn)

Ablesung nach	Harn Nr.									
	1	2	3	4	5	6	7	8	9	10
½ Stunde E	0,38	0,12	0,36	0,20	0,49	0,25	0,35	0,47	0,47	0,20
16 Stdn E	0,38	0,13	0,36	0,19	0,50	0,25	0,37	0,49	0,48	0,20
26 » E	0,39	0,14	0,38	0,20	0,50	0,27	0,36	0,50	0,49	0,21

durchgeführt wird, z. B. 1 ccm Urochromlösung + 19 ccm Aq. dest., 2 ccm Urochromlösung + 18 ccm Aq. dest. usw. Es ergibt sich dann eine Reihe von Urochromlösungen, deren relative Urochromkonzentration sich mit den Zahlen 1, 2 usw. ausdrücken lässt.

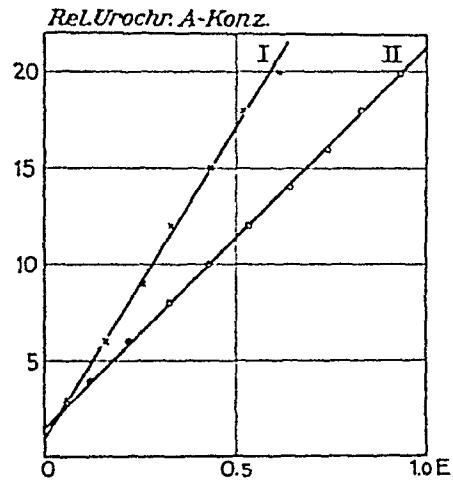

Abb. 1. *Das Verhältnis Urochrom-A-Konzentration/Extinktionswert.*
Kurve I: Lösung aus »Histidinversuch».
Kurve II: Meerschweinchenharn.

Wie aus Abb. 1 hervorgeht, besteht eine direkte Proportionalität zwischen Konzentration und Extinktionswerten.

In der eingangs erwähnten Arbeit (Ekman 1940) konnte eine solche direkte Proportionalität nicht nachgewiesen werden. Mit dem jetzt angewandten Verfahren ist dies indessen stets gelungen, vorausgesetzt, dass die Extinktionswerte nicht 1,4—1,5 übersteigen. Da indessen das Pulfrich-Photometer keine exakten Ablesungen so hoher Extinktionswerte erlaubt, spielt die Einschränkung, die also bezüglich des Konzentrationsbereiches, innerhalb dessen des Beersche Gesetz gilt, zu machen

TABELLE 10.

Zusatz von Askorbinsäure zum Harn bewirkt eine gewisse Steigerung der Urochrom-A-Werte.

Nachdem die Askorbinsäure den Harnen zugesetzt worden war, blieben diese drei Tage unter Toluol stehen. Überall Mittelwert aus Doppelbestimmungen.

Harn von:	Menschen E	Meerschweinchen	
		E(a)	E(b)
100 ccm Harn + 100 mg Ask.	0,325	0,75	
+ 50 » »	0,30	0,635	0,24
— — —	0,305	0,575	0,255

TABELLE 11.

Einwirkung des Zusatzes von Hydrochinon und Brenzkatechin auf den Extinktionswert. (Eigener Harn)

Zusatz in mg/20 ccm Harn	0	0,5	1	3	6	10
E bei Zusatz von Hydrochinon	0,08	0,08	0,15	0,16	0,24	0,32
Brenzkatechin	0,08	0,08	0,11	0,21	0,19	0,15

TABELLE 12.

Der Urochrom-A-Gehalt des Harns verändert sich bei Aufbewahrung nur wenig.

Versuche mit Meerschweinchenharn, der 3 Tage lang gesammelt und dann teils sofort und teils nach wechselnd langer Zeit analysiert wurde.

Harn Nr.	Bestimmung nach			
	Unmittelbar	1 Tag	2 Tagen	3 Tagen
1 E	0,50	0,47	0,47	
2 E	0,70	0,69	0,69	
3 E	0,62	0,58	0,55	
4 E	0,22	0,16	0,18	
5 E	0,33			0,32

ist, praktisch keine Rolle. Durch geeignete Verdünnung lassen sich selbstverständlich auch diese konzentrierten Lösungen bestimmen.

In der vorliegenden Arbeit wird die Urochrom-A-Bestimmungsmethode an Urin angewandt werden, der wechselnde Mengen Askorbinsäure und in gewissen Fällen Polyphenole enthält. Von prinzipieller Bedeutung ist es dabei, festzustellen, ob der Zusatz dieser Stoffe zum Harn die Urochromwerte ändert. Aus Tabelle 10 geht hervor, dass bei hohen Askorbinsäurekonzentrationen im Harn die Tendenz steigender Urochrom-A-Werte auftritt. Dies dürfte sich dadurch erklären lassen, dass zyklische Verbindungen im Harn durch Askorbinsäure unter Entstehung von Urochrom A oxydiert werden, analog den Ergebnissen der Versuche in vitro in Kap. III. Tabelle 11 zeigt, dass wenn Brenzkatechin oder Hydrochinon zu einem Harn gesetzt werden (nach dem Zusatz blieb der Harn 24 Stdn stehen), diese Polyphenole erhöhte Urochrom-A-Werte verursachen. Es muss aber betont werden, dass die im Harn ausgeschiedenen Polyphenole grösstenteils mit Säuren gepaart (NEUBAUER-HUPPERT, 1913) und damit auch stabilisiert worden sind und wie eine Untersuchung in Kap. IV erweist, wurde die Polyphenolausscheidung nach Einverleibung von täglich 50 mg Hydrochinon nur auf ungefähr 3 mg erhöht (berechnet als Hydrochinon). Nach Tabelle 11 stieg aber der Urochrom-A-Extinktionswert nur von 0,08 bis 0,16 bei einem Zusatz von 3 mg Hydrochinon. Es dürfte also angenommen werden, dass die Veränderungen der Urochrom-A-Werte in vivo durch Zufuhr von zyklischen Verbindungen das Ergebnis eines chemischen Vorgangs sei, der im Organismus und nicht im Harn stattgefunden hat.

Bei Urochrombestimmungen im Harn muss dieser während kürzerer oder längerer Zeit gesammelt und aufbewahrt werden. Der Harn wurde stets unter einer etwa cm-dicken Schicht von Toluol aufbewahrt, und wie aus Tabelle 12 hervorgeht, erfährt unter diesen Bedingungen die Urochrom-A-Konzentration keine Veränderungen, welche die Beurteilung des Resultats beeinflussen können.

Die Spezifität der Methode.

Mit Hilfe des hier angegebenen Verfahrens lassen sich selbstverständlich alle durch Kupferazetat fällbaren Farbstoffe, die in alkalischem und saurem Milieu haltbar sind und mit dem Filter S47 eine Extinktion liefern, bestimmen. In der vorliegenden Arbeit ist die Entstehung von durch Kupferazetat fällbaren Farbstoffen unter folgenden Verhältnissen untersucht worden:

1. In vivo bei wechselnder C-Vitaminzufuhr und bei wechselnder Zufuhr zyklischer Verbindungen.

2. Bei Versuchen in vitro mit zyklischen Verbindungen und Askorbinsäure.

Soweit ich in der mir zugänglichen Literatur habe feststellen können, sind im normalen Harn nur Urochrom A und die Purinstoffe durch Kupferazetat fällbar. Die Purinstoffe sind indessen farblos, können also die Urochrombestimmung nicht beeinflussen. Wahrscheinlich ist es jedoch möglich, beispielsweise in Tierversuchen mit der Kost Farbstoffe einzuverleiben, die im Harn ausgeschieden werden und zu hohe Urochrom-A-Werte veranlassen können. Ferner hat es sich gezeigt, dass man zu hohe Urochrom-A-Werte erhält, wenn der Harn mit Blut verunreinigt ist, z. B. aus Darmblutungen skorbutischer Meerschweinchen.

Um die mittels der Methode gefundenen Urochrom-A-Werte aus den Versuchen in vivo wie in vitro vergleichbar zu gestalten, bin ich wie folgt vorgegangen:

1. In sämtlichen Tierversuchen wurde ein und dieselbe Kost gereicht, die an und für sich farblos ist. (Siehe S. 92.)

2. Da die Bestimmungsmethode auf einer Fällungsreaktion fusst, kann man dieselbe Fällungsreaktion unter denselben Verhältnissen wie in der quantitativen Bestimmungsmethodik anwenden, um den in jedem Falle untersuchten Farbstoff zu isolieren. Dies ist auch geschehen, und wie aus Kap. VII hervorgeht, können die »Urochrome», die unter wechselnden Verhältnissen mit dem Harn ausgeschieden oder bei Versuchen in vitro gebildet werden, durch eine spektrographische Untersuchung nicht voneinander unterschieden werden, und sie verhalten sich gegenüber Zusätzen von Ammoniak und Salzsäure gleich. Wenn auch eine vorläufige Untersuchung gewisser chemischer Verhältnisse ergibt, dass die Farbstoffe nicht in sämtlichen Fällen identisch sind, muss man doch annehmen, dass sie eng miteinander verwandt sind und dass die geschilderte kolorimetrische Bestimmungsmethode reproduzierbare Werte liefert, die zur Grundlage von Studien über die Verhältnisse dieser Farbstoffe in vivo und in vitro gemacht werden können.

Die Zuverlässigkeit der Methode.

Um den mittleren Fehler der Einzelbestimmung zu ermitteln, machte ich 10 Bestimmungen im selben Harn mit vollständigem Analysengang. Die Extinktionswerte waren folgende:

0,897 0,890 0,893 0,885 0,892 0,913 0,900 0,903 0,897 0,899

Der mittlere Fehler des Mediums beträgt ± 0,0023, die Dispersion ± 0,0073.

II. Quantitative Bestimmung freier und gebundener Salizylsäure.

Die quantitative Bestimmung von Salizylsäure ist in sehr zahlreichen Untersuchungen bearbeitet worden. Das reiche Schrifttum wolle man bei Thoburn und Hanzlik (1915), Loberg (1926) und Vartiainen (1934) nachschlagen. Hier sei nur erwähnt, dass die allgemeine Ansicht dahinzugehen scheint, dass Salizylsäure, die Menschen oder Tieren einverleibt wird, teils unverändert und teils gebunden, und zwar letzterenfalls hauptsächlich als Salizylursäure, ausgeschieden wird. Nachdem es Holmes (1926) und Quick (1933) gelungen ist, Salizylursäure aus Harn zu isolieren und mit völliger Sicherheit zu identifizieren, dürfte Hanzliks (z. B. 1930) negativen Versuchen keine Bedeutung mehr beigemessen werden können. Gewöhnlich scheint man die kolorimetrischen Methoden den ursprünglichen gravimetrischen und den später ausgearbeiteten massanalytischen, die sich auf die Reaktionen der Salizylsäure mit Jod und Brom gründen, vorzuziehen.

Als Reagens bei der kolorimetrischen Bestimmung ist hauptsächlich Ferrichlorid benutzt worden. In sehr eingehenden Untersuchungen hat Vartiainen (1934) zeigen können, dass die Farbreaktion zwischen Salizylsäure und Ferrichlorid sich gut als Grundlage eines quantitativen Bestimmungsverfahrens eignet.

Bei Bestimmungen in Urin wirken indessen die Farbstoffe und auch andere Bestandteile des Harns in erheblichem Grade störend ein, weshalb man im allgemeinen die Salizylsäure aus dem Harn zu isolieren versucht und dann die Bestimmung in einer reinen wässrigen Lösung gemacht hat. Vartiainen (1934) extrahierte die Salizylsäure mit Äther, Hanzlik (1915) und

HOLMES (1926) bedienten sich eines Destillationsverfahrens. Sowohl VARTIAINEN als HOLMES geben indessen an, dass bei ihren Verfahren Salizylverluste auftreten, die im allgemeinen 10 % der zur Kontrolle zugesetzten Salizylsäuremengen ausmachen.

Die schon vorliegenden Bestimmungsmethoden dürften also mit ernsten methodologischen Fehlern behaftet sein. Hierzu kommt, dass sämtliche Verfahren, die eine Bestimmung auch der gebundenen Salizylsäure gestatten, mit einer Hydrolyse durch Kochen mit Säure recht grosser Harnmengen in mit Rücklaufkühler versehenen Kolben arbeiten. Da die Hydrolyse 3 (VARTIAINEN) bis 12 (BENZINGER und WYRSCH, 1933) Stunden beansprucht, dürften daher immer nur sehr wenige Proben gleichzeitig analysiert werden können.

Die Schwierigkeiten, eine einfache und zuverlässige Methodik zu finden, spiegeln sich in der Tatsache, dass man bis heute noch kaum weiss, ob Salizylsäure im lebenden Organismus abgebaut werden kann oder ob die Entgiftung nur dadurch geschieht, dass die Säure mit Glykokoll, Schwefelsäure oder Glykuronsäure gepaart wird.

Die Ausarbeitung einer neuen Salizylsäurebestimmungsmethode erschien daher erwünscht.

Das Verfahren sollte folgende Forderungen erfüllen: einfache Hydrolyse der gebundenen Salizylsäure, gleichzeitige Bearbeitung einer grossen Anzahl von Proben, quantitatives Wiederfinden der den Versuchslösungen zugesetzten Salizylsäure.

Ein gangbarer Weg erschien der, die Hydrolyse durch Kochen auf dem Wasserbad von kleinen Lösungsmengen in Probiergläsern vorzunehmen, die mit einer einfachen Kondensationsvorrichtung vom selben Typus versehen sind, wie er bei Kochen in Kjeldahlkolben (ANDERSEN und JENSEN, 1926) zur Anwendung kommt. Es ist seit langem bekannt, dass die Salizylsäure aus ihrer Lösung mit Chloroform quantitativ ausgeschüttelt werden kann. Dies scheint zur Grundlage einer bei der Salizylsäurebestimmung bisher nicht angewandten Methode gemacht werden zu können: Ein Teil der Probe wird mit dem Extraktionsmittel geschüttelt, und man kann auf diese Weise, wenn das Extraktionsmittel spezifisch für Salizylsäure ist, zwei Portionen erhalten, die sich nur so voneinander unterscheiden, dass die eine

Salizylsäure enthält und die andere nicht. Versetzt man dann beide Portionen mit dem Reagens, so wird der Farbunterschied einzig durch die Reaktion mit der Salizylsäure bedingt sein. Sonstige Stoffe, die eventuell mit dem Ferrichlorid reagieren können, finden sich in beiden Portionen und können die Bestimmung nicht stören.

Chloroform, das in diesem Verfahren als Extraktionsmittel benutzt worden ist, nimmt indessen auch gewisse Farbstoffe aus dem Harn auf: Urochrom B, Gallfarbstoffe und Uroerythrin. Bei Verwendung des ZEISS'schen PULFRICH-Photometers zur Kolorimetrie kann man jedoch mit einem Filter arbeiten, das die Eigenfarbe dieser Stoffe vollständig eliminiert. Da die betreffenden Stoffe, soweit bekannt, nicht mit Ferrichlorid reagieren, spielt ihre gleichzeitige Extraktion keine Rolle.

Methodik.

Prinzip: I. *Freie Salizylsäure.* Der Harn wird angesäuert und in zwei Portionen geteilt, von denen die eine mit Chloroform geschüttelt wird. Dann werden beide Portionen mit Ferrichlorid versetzt und gegeneinander kolorimetriert.

II. *Gesamtsalizylsäure* (freie + gebundene). Der Harn wird durch Kochen auf dem Wasserbad mit Salzsäure hydrolysiert. Im übrigen wird wie bei der Bestimmung freier Salizylsäure verfahren.

Ausführung.

I. *Freie Salizylsäure.* 1 ccm Harn wird in einem Probiergläschen mit 5 ccm 1n Salzsäure und 4 ccm Aq. dest. versetzt. Nachdem der Inhalt geschüttelt worden ist, werden 2 ccm in ein anderes Probierglas gebracht und darin stehengelassen. Die restlichen 8 ccm werden 5 Min. lang mit etwa der gleichen Menge Chloroform im Schüttelapparat geschüttelt. Diese Extraktion wird im ganzen 3mal wiederholt. (Das Chloroform muss mit etwa 1n HCl ½ Stunde lang geschüttelt sein, damit nicht die zu extrahierende Lösung irgendwelcher Salzsäure beraubt wird. Chloroform und wässrige Lösung lassen sich am einfachsten in der Weise scheiden, dass man das Chloroform mit einer Wasserstrahlpumpe aufsaugt, die mit einer Auffangflasche für das Chloroform verbunden ist. Nach der letzten Extraktion empfiehlt es sich zu filtrieren.)

2 ccm des Filtrats werden dann in ein Probierglas gebracht und, ebenso wie die restlichen 2 ccm der nicht extrahierten Probelösung, mit 5 ccm 20 % Ferrichloridlösung versetzt.

Die beiden Lösungen werden im ZEISS'schen PULPHRICH-Photometer mit dem Filter S57 und in der 10 mm-Küvette gegeneinander kolorimetriert.

II. *Gesamtsalizylsäure.* In einem Probierglas werden 2 ccm Harn mit 5 ccm Salzsäure (150 ccm Aq. dest. + 850 ccm reine konz. HCl) versetzt. In der Mündung des Probierglases wird eine Glaskugel mit Stiel angebracht (ANDERSEN und JENSEN, 1926), worauf man das Probierglas 3 Stunden im kochenden Wasserbad stehenlässt. Nachdem das Gemisch abgekühlt ist, wird mit Aq. dest. auf 20 ccm aufgefüllt und anschliessend filtriert. Von dem Filtrat werden etwa 10 ccm durch viermaliges Schütteln mit Chloroform extrahiert, der Rest bleibt stehen.

2 ccm der nicht extrahierten Probelösung und 2 ccm der extrahierten Lösung werden in 2 Probiergläsern mit je 1 ccm 16 % NaOH und 5 ccm 20 % Ferrichlorid versetzt. Dann kolorimetriert man wie bei der freien Salizylsäure. (Nach Zusatz der Natronlauge müssen etwa 4,0 ccm 0,1n Natronlauge für eine vollständige Neutralisation der Lösung nötig sein.)

Kalibrierung der Methode und Berechnung der Ergebnisse.

Die Faktoren, die in erster Linie die Formel zur Berechnung des Ergebnisses beeinflussen, sind die Konzentrationen der Lauge und der Säure (siehe unten). Es dürfte bedeutend einfacher sein, nach jeder Neubereitung von Lauge und Säure die Methode gegen bekannte Salizylsäurelösungen zu stellen, als jedesmal Lauge und Säure von einer gewissen exakten Konzentration zu bereiten.

Beispiel für Kalibrierung und Berechnung des Ergebnisses: 200 mg Natriumsalizylat wurden in 100 ccm dest. Wasser gelöst und in dieser Lösung 5 Bestimmungen nach der Methodik teils für freie und teils für Gesamtsalizylsäure gemacht. Der mittlere Extinktionswert bei Bestimmung freier Salizylsäure war 0,43. Ein Extinktionswert von 1,0 entspricht also 4,65 mg Natriumsalizylat pro ccm Probelösung. Die Berechnungsformel ist daher: $E \times 4{,}65 \times 100 =$ Natriumsalizylat in mg%. — Der mittlere Extinktionswert bei der Bestimmung von Gesamtsalizylsäure war 0,21. Ein Extinktionswert von 1,0 entspricht also 9,52 mg Natriumsalizylat pro ccm Probelösung. Die Berechnungsformel ist also: $E \times 9{,}52 \times 100 =$ Natriumsalizylat in mg%.

In dieser Form ist die Methodik brauchbar für Bestimmungen in Lösungen mit 25—500 mg% freier Salizylsäure und 50—1000 mg% Gesamtsalizylsäure (als Natriumsalizylat berechnet).

In erster Linie durch Benutzung anderer Küvetten, ferner durch einfache Veränderungen im Verhältnis zwischen der Ausgangsmenge der Probelösung und der übrigen Lösungen lässt sich das Verfahren sowohl bei konzentrierteren als bei schwächeren Salizylsäurelösungen verwenden.

Methodologische Belege.

1. *Extraktion.*

In den früheren Arbeiten, in denen das Extraktionsprinzip angewandt worden ist, hat man stets aus einer angesäuerten Lösung extrahiert. Wie Tabelle 13 zeigt, erfolgt die Extraktion aus einer reinen Natriumsalizylatlösung mit Ansäuerung etwas schneller als ohne solche, doch dürfte der Unterschied in dieser Beziehung kaum praktische Bedeutung haben. Wenn man mit Urin arbeitet, wird indessen die Emulsionsbildung zwischen Chloroform und Lösung durch die Ansäuerung erheblich behindert, und vor allem ist es weit leichter, eine klare Lösung zu erhalten, wenn man Salzsäure zusetzt. Aus der Tabelle geht ferner hervor, dass die Extraktion freier bzw. der Gesamtsalizylsäure schon nach 2- bzw. 3maligem Ausschütteln vollständig ist. Das dritte und vierte Schütteln, wie es nach der Methodik ausgeführt wird, dürfte also eine vollständige Extraktion gewährleisten.

TABELLE 13.

Die zur vollständigen Extraktion der Salizylsäure notwendige Anzahl von Einzelextraktionen.

Anzahl Extraktionen		1	2	3	4	5
Methode für:						
Freie Salizylsäure	E	0,34	0,385	0,39	0,395	0,39
Freie Salizylsäure ohne Zusatz von HCl	E	0,025	0,18	0,19	0,19	0,19
Totalsalizylsäure	E	0,68	0,725	0,77	0,785	0,785

2. *Von den Stoffen, die mit Chloroform aus dem Harn extrahiert werden, liefert einzig die Salizylsäure mit dem Filter S57 eine erkennbare Farbe mit Ferrichlorid.*

Bei Bestimmungen in mehreren Harnen von Meerschweinchen und Menschen, die keine Salizylsäure enthielten, war die Extinktion im allgemeinen = 0 (höchster beobachteter Wert = 0,02). Die Methode dürfte also als für die Salizylsäure spezifisch gelten können.

3. *Die Ferrichloridkonzentration.*

Die hier mitgeteilte Methode unterscheidet sich von den früheren u. a. dadurch, dass das Ferrichlorid einer stark sauren Lösung zugesetzt wird. Mehrere Autoren haben hervorgehoben, dass die Farbe, die sich bei der

Reaktion zwischen Salizylsäure und Ferrichlorid bildet, beim Hinzukommen von Salzsäure verschwindet, dass man sie aber zurückerhalten kann, wenn man Ferrichlorid in starkem Überschuss hat. Die Neutralisation musste bei den früheren Verfahren mit neutralen oder schwach sauren Lösungen mittels einer schwierigen Einstellung auf einen bestimmten pH-Wert geschehen, und leichte Veränderungen dieses Wertes dürften merkbare Schwankungen der Farbstärke ergeben haben. Einfacher erschien es daher, mit einer entschieden sauren Lösung zu arbeiten, wobei kleine Änderungen der Azidität keine grössere Rolle spielen. Wie aus Tabelle 14 hervorgeht, erzielt man mit einer 20 %igen Ferrichloridlösung bei der hier angewandten sauren Lösung eine maximale Extinktion. Geringere Schwankungen der Ferrichloridkonzentration spielen, wie man sieht, keine Rolle.

4. *Wahl des Filters.*

Wie man aus Tabelle 15 ersieht, erzielt man mit dem Filter S53 eine maximale Extinktion bei Bestimmung in reiner Natriumsalizylatlösung. Die Extinktion mit S57 ist nicht so viel niedriger, dass es für die Empfindlichkeit der Methode belangvoll sein könnte, und wir wählen dieses Filter, um nach Möglichkeit einen störenden Einfluss vonseiten der Harnfarbstoffe auszuschliessen.

5. *Die Haltbarkeit der Farbe beim Kolorimetrieren.*

Tabelle 16 zeigt, dass die Reaktion Salizylsäure-Ferrichlorid sich schnell auf eine konstante Farbe einstellt, die besonders bei der Bestimmung von Gesamtsalizylsäure sehr haltbar ist. Bei freier Salizylsäure erhält man nach 24 Stunden eine gewisse Steigerung der Extinktion, die wahrscheinlich darauf beruht, dass in der sauren Lösung eine Hydrolyse gebundener Salizylsäure stattfindet.

6. *Kochzeit.*

Tabelle 17 zeigt, dass die Hydrolyse der gebundenen Salizylsäure im allgemeinen nach 2stündigem Kochen auf dem Wasserbad, stets aber nach 3 Stunden abgeschlossen wurde. Im Gegensatz zu VARTIAINENS (1934) Versuchsanordnung kann man hier, trotz fortgesetzten Kochens, keine Zerstörung der Salizylsäure feststellen.

7. *Die Salzsäurekonzentration bei der Hydrolyse.*

Tabelle 18 zeigt, dass bei recht stark verschiedenen Salzsäurekonzentrationen gleiche Hydrolysenergebnisse erzielt werden. Hier ist eine relativ hohe Salzsäurekonzentration gewählt worden, doch ist die konzentrierte Säure, mit der zu arbeiten schwierig ist, vermieden worden.

TABELLE 14.

Maximale Extinktion wird mit einer 20 % Ferrichloridlösung erzielt.

In jedem Versuch wurde so viel Aq. dest. beigegeben, dass das Gesamtvolumen von Ferrichlorid und dest. Wasser 5 ccm war.

ccm 20 % $FeCl_3$	0,2	0,3	0,4	0,5	1	2	3	4	5
Best. von freier Salizylsäure E	0,37	0,44	0,48	0,53	0,60	0,65	0,70	0,70	0,72
Best. von Totalsalizylsäure E	0,20	0,25	0,29	0,33	0,40	0,46	0,50	0,53	0,53

TABELLE 15.

Die Extinktionswerte mit verschiedenen Filtern bei Bestimmung reiner Salizylsäurelösungen.

Filtrum	S47	S50	S53	S57	S61
Bestimmung von					
Freier Salizylsäure E	0,57	0,66	0,71	0,61	0,37
Totalsalizylsäure E	0,46	0,53	0,59	0,52	0,32

TABELLE 16.

Die Haltbarkeit der Kolorimetriefarbe.

a. Bestimmung von freier Salizylsäure.

Harn Nr.		1	2	3	4	5
Ablesung nach ½ Stunde	E	0,46	0,56	0,75	1,005	1,235
nach 15 Stunden	E	0,465	0,555	0,75	1,01	1,22
nach 24 Stunden	E	0,49	0,56	0,75	1,04	1,26

b. Bestimmung von Totalsalizylsäure.

Harn Nr.		1	2	3	4	5
Ablesung nach ½ Stunde	E	0,26	0,35	0,44	0,51	0,575
nach 15 Stunden	E	0,26	0,35	0,445	0,52	0,58
nach 24 Stunden	E	0,27	0,35	0,445	0,52	0,58

TABELLE 17.

Bestimmung der Hydrolysendauer.

Kochzeit in Stunden:		1	2	3	4	7
Meerschweinchenharn						
Nr. 1	E	0,105	0,15	0,165	0,165	
Nr. 2	E	0,205	0,225	0,225	0,23	
Nr. 3	E	0,215	0,25	0,25	0,255	
Nr. 4	E	0,265	0,295	0,30	0,30	
Nr. 5	E		0,50	0,52	0,52	0,52
Eigener Harn	E		0,165	0,16	0,165	
Salizylsäurelösung	E		0,52			0,52

TABELLE 18.

Die Salzsäurekonzentration bei der Hydrolyse.

In jedem Versuch wurde so viel Aq. dest. zugesetzt, dass beim Kochen das Gesamtvolumen von Salzsäure und dest. Wasser 5 ccm betrug. Nach dem Kochen wurde im Zusammenhang mit der Verdünnung so viel Salzsäure zugesetzt, dass die gesamte Menge Salzsäure in jedem Versuch 5 ccm war. Kochzeit 1 Stunde. Eigener Harn.

ccm konz. HCl	1	2	3	4	5
E	0,09	0,11	0,13	0,13	0,135

8. *Bei Zusatz bekannter Mengen von Natriumsalizylat zum Harn werden diese quantitativ wiedergefunden. Es herrscht eine direkte Proportionalität zwischen Extinktionswerten und Salizylsäuremengen.*

Für eine Serie von Harnen von Meerschweinchen, die Natriumsalizylat bekommen hatten, wurden die Extinktionswerte bei Salizylsäurebestimmung festgestellt. Danach wurden diesen Harnen bekannte Mengen Natriumsalizylat zugesetzt, ferner wurden dieselben Mengen Natriumsalizylat in den Harnportionen entsprechenden Volumina dest. Wassers gelöst, worauf auch in diesen Lösungen Bestimmungen ausgeführt wurden. Falls die Methode korrekte Werte liefert, muss der Unterschied zwischen dem Extinktionswert bei Bestimmung in einem Harn nach Zusatz von Natriumsalizylat und dem Extinktionswert bei Bestimmung im selben Harn ohne Zusatz gleich dem Extinktionswert bei Bestimmung in der entsprechenden Wassermenge mit demselben Natriumsalizylatzusatz sein.

Diese Extinktionswerte müssen ferner der Natriumsalizylatmenge direkt proportional sein. Wie aus Abb. 2 hervorgeht, sind diese Forderungen auch erfüllt, und zwar gilt dies unabhängig davon, ob man nach der Methode für freie oder der für die Gesamtsalizylsäure verfährt.

Gegen diesen Kontrollversuch lässt sich der Einwand erheben, es sei nicht versucht worden, bekannte Mengen einer gebundenen Form von Salizylsäure, z. B. Salizylursäure, wiederzufinden. Die Vorschriften für die Hydrolyse sind indessen so gewählt worden, dass sie eine maximale Freimachung gebundener Salzsäure gewährleisten, was, wie in Tab. 17

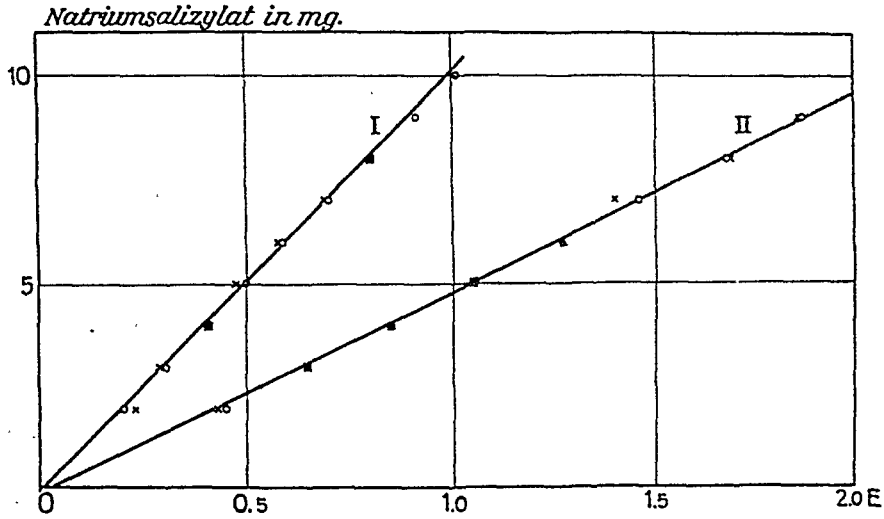

Abb. 2. *Das Verhältnis Natriumsalizylatkonzentration/Extinktionswert.*

Kurve I: Bestimmung von Gesamtsalizylsäure. Kurve II: Freie Salizylsäure. o = Bestimmung in Wasserlösung, x = in Harn.

ersichtlich, dadurch gezeigt wird, dass fortgesetztes Kochen keine höheren Extinktionswerte mit sich bringt.

Schliesslich dürfte folgender Versuch zeigen, dass man mit der hier angewandten Methodik korrekte Ergebnisse auch bei der Bestimmung von Gesamtsalizylsäure erzielt:

Im Selbstversuch wurden täglich 500 mg Natriumsalizylat eingenommen. Nach einer Vorbereitung von 2 Tagen wurden täglich während einer Zeit von 15 Tagen Bestimmungen der ausgeschiedenen Tagesmengen von Gesamtsalizylsäure gemacht. Der Mittelwert, als Natriumsalizylat berechnet, der täglichen Ausscheidung war 500,3 mg.

(Der Versuch dürfte auch für Probleme im Zusammenhang mit der Pharmakologie der Salizylsäure von Interesse sein: Nach den Versuchen an dieser einzelnen Versuchsperson zu urteilen, wird Salizylsäure bei der verabreichten verhältnismässig niedrigen Tagesgabe weder aufgespeichert noch verbrannt.)

9. *Zuverlässigkeit der Methode.*

Um den mittleren Fehler der Einzelbestimmung herauszufinden, wurden 10 Bestimmungen im gleichen Harn mit vollständigem Analysengang gemacht. Bei der Bestimmung freier Salizylsäure wurden folgende Werte gefunden:

0,418 0,410 0,412 0,413 0,409 0,418 0,409 0,414 0,409 0,417

Der mittlere Fehler des Mediums beläuft sich auf ± 0,0011, die Dispersion auf ± 0,0035.

Die Extinktionswerte bei Bestimmung der Gesamtsalizylsäure waren:

0,208 0,208 0,203 0,203 0,200 0,208 0,198 0,204 0,201 0,197

Der mittlere Fehler des Mediums beträgt ± 0,0012, die Streuung ± 0,0039.

(Bei dem hier beschriebenen Verfahren benötigt man zur Bestimmung der freien Salizylsäure für die Analyse von 20 Proben 2—3 Stunden, bei der Bestimmung der Gesamtsalizylsäure, wenn man die für die Hydrolyse benötigte Zeit abzieht, 3—4 Stunden.)

III. Quantitative Bestimmung von Indol.

A. Im Harn.

Für Stoffe, die eine Indolgruppe enthalten, sind mehrere Farbreaktionen vorhanden. Es lassen sich folgende Hauptgruppen unterscheiden:

1. Mit Natrium-β-naphtochinon-4-sulfonat.
2. Mit dem Folinschen »Phenolreagens» (Phosphormolybdensäure).
3. Mit Salzsäure oder Schwefelsäure oder einem Gemisch aus diesen nebst einem aliphatischen oder aromatischen Aldehyd.

Diese Farbreaktionen sind zur Grundlage mehrerer Methoden zur quantitativen Bestimmung von Indol in den Fäzes gemacht worden (z. B. Bergeim 1917), dagegen, soweit ich sehen kann, nur für ein Verfahren zur Indolbestimmung im Harn, nämlich nach Forbes und Neale (1935).

Die Methodik ist folgende: 100 ccm Harn werden mit Weinsäure angesäuert und destilliert. Das Destillat wird alkalisiert und noch einmal destilliert. In diesem zweiten Destillat erhält man mit KOH und Natrium-β-naphtochinon-4-sulfonat eine rote Farbe mit eventuell anwesendem Indol. Der Farbstoff wird mit Chloroform ausgeschüttelt. Nach Verdünnung der Chloroformlösung mit alkalischem Alkohol entsteht eine grünblaue Farbe, die kolorimetriert wird.

Wie die Autoren angeben, ist die Farbreaktion mit dem von ihnen verwendeten Reagens spezifisch für Indol, und es lassen sich schon Mengen von 5 γ in 100 ccm Harn bestimmen. Das Verfahren ist aber sehr zeitraubend und schwierig und liefert, wie FORBES und NEALE angeben, keine ganz quantitativen Resultate.

Für die Bestimmung von Indol im Harn, der ja normalerweise Phenole enthält, dürfte das FOLINsche Reagens weniger geeignet sein. Natrium-β-naphtochinon-4-sulfonat ist gegenwärtig leider nicht erhältlich, weshalb die Kombination Mineralsäure-Aldehyd als Reagens gewählt werden muss.

Farbreaktionen mit diesen Stoffen sind zum Gegenstand einer eingehenden Untersuchung von LIEBEN und POPPER (1926) gemacht worden, mit dem Ziele, deren Eignung für quantitative Bestimmungsmethoden zu erforschen. Die genannten Autoren stellen zusammenfassend fest: »Die verschiedenen Farben, die durch Zusammenbringen der angeführten Verbindungen (heterozyklischer Verbindungen) mit einer Reihe von Aldehyden und konzentrierter H_2SO_4 auftreten, richten sich nach ihrer qualitativen und quantitativen, im Kolorimeter feststellbaren Farbstärke, nach der heterozyklischen Verbindung, wenn der Aldehyd im (molaren) Überschuss ist, nach dem Aldehyd, wenn dies für die heterozyklische Verbindung zutrifft.» Ferner weisen sie darauf hin, dass in naheliegenden Konzentrationen von Aldehyd und heterozyklischer Verbindung ein Störungsgebiet vorhanden ist, in welchem die beiden möglichen Reaktionen nebeneinander vor sich gehen. Unter den untersuchten Aldehyden gehört das Vanillin zu denen, die vom quantitativen Gesichtspunkt aus die besten Resultate lieferten.

BRUMMER (1940) hat ein quantitatives Bestimmungsverfahren für einen anderen Stoff mit einer Indolgruppe ausgearbeitet, nämlich für das Tryptophan, und in vorbereitenden Versuchen hat er die Eignung verschiedener Aldehyd-Säure-Reaktionen untersucht. Die besten Ergebnisse wurden mit H_2SO_4 und Vanillin erzielt. Im Falle des Tryptophans konnte die Empfindlichkeit durch einen Zusatz kleiner Mengen Natriumsulfid bedeutend gesteigert werden.

Zur Bestimmung von Indol im Harn ist jedoch die Reaktion mit Vanillin und konzentrierter Schwefelsäure nicht ohne weite-

res brauchbar, da Harn mit Säure allein eine starke Farbe liefert, die der mit Indol sich ergebenden ähnlich ist.

Indol ist in Chloroform leichtlöslich, weshalb folgendes Prinzip für eine quantitative Bestimmungsmethode brauchbar erschien: der Harn wird in zwei Portionen geteilt, von denen die eine mit Chloroform geschüttelt wird, während die andere unbehandelt bleibt. Dann werden beide Portionen mit Schwefelsäure und Vanillin versetzt. Der auftretende Farbunterschied richtet sich nach der Indolkonzentration, denn da die eine Portion mit Chloroform geschüttelt worden ist, enthält die eine Harnportion Indol und die andere nicht.

Die Farbreaktion mit Vanillin und Schwefelsäure ist nicht spezifisch für Indol; wie schon erwähnt, vollzieht sich die gleiche Reaktion mit Tryptophan, ausserdem aber auch mit Pyrrol und Thiophen. Laut Untersuchungen von VAUGHAN (1936) dürfte der Harn normalerweise Tryptophan enthalten, doch in sehr geringfügigen Mengen. Tryptophan ist indessen in Chloroform unlöslich, es vermag also eine Bestimmungsmethode nach dem obigen Prinzip nicht zu stören, und Pyrrol und Thiophen dürften unter normalen Verhältnissen nicht im Harn vorhanden sein. Es liegen keine Angaben vor, ob Indoxyl die gleiche Reaktion ergibt, und da Indoxyl in Chloroform leichtlöslich ist, wird diese Frage bei der experimentellen Prüfung der Methode untersucht.

Vorläufige Methodik.

5 ccm Harn werden in einem Reagenzglas mit 5 ccm dest. Wasser versetzt. 0,5 ccm des Gemischs werden in einem Reagenzglas abgestellt (Portion I). Der Rest wird dreimal in der Schüttelmaschine mit Chloroform geschüttelt. Von dem ausgeschüttelten Harn wird 0,5 ccm in ein Reagenzglas gebracht (Portion II).

Beide Harnportionen werden mit je 0,1 ccm einer 0,15 % Vanillinlösung und 4 ccm 60 % Schwefelsäure versetzt. Anschliessend Kolorimetrieren in den 1 cm-Küvetten und mit Filter S50 im ZEISSschen PULFRICH-Photometer. Bei Zusatz wechselnder Indolmengen zu verschiedenen Harnen wurde, wie aus Abb. 3 ersichtlich, gute Übereinstimmung zwischen Extinktionswert und Indolkonzentration gefunden. Die Konstante für dieses Verhältnis ist indessen, wie aus der Abbildung hervorgeht, für verschiedene Harne verschieden.

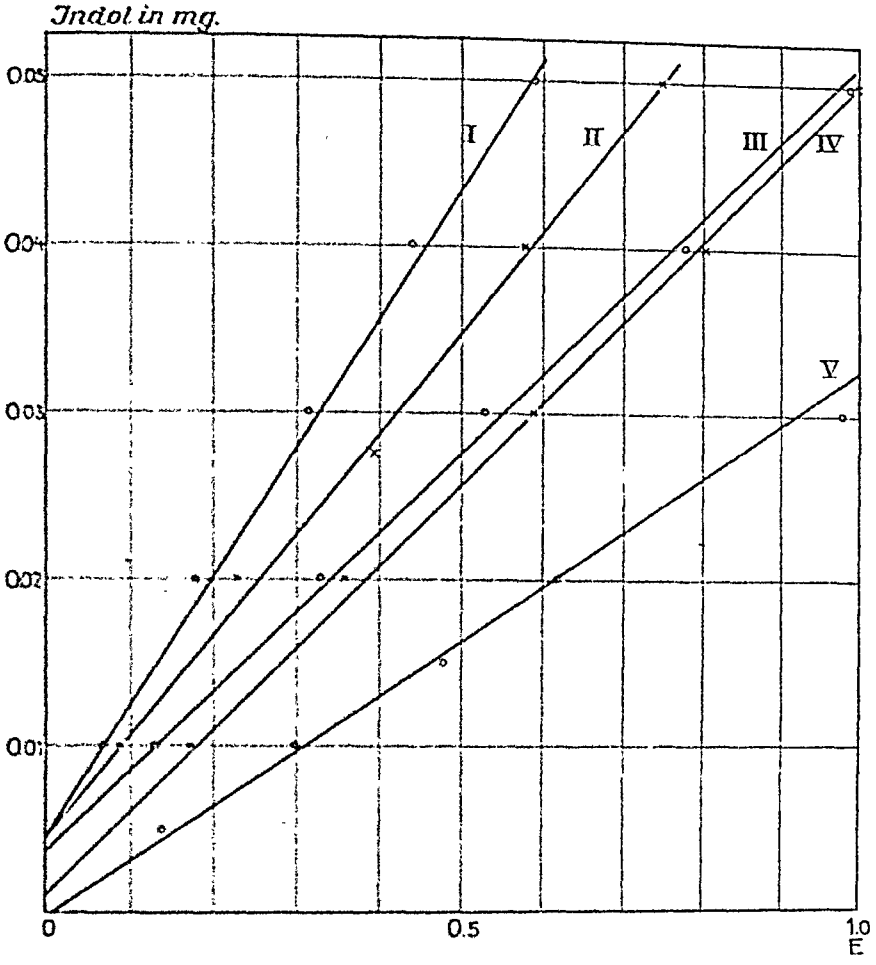

Abb. 3. *Das Verhältnis Indolkonzentration/Extinktionswert.*

Bekannte Indolmengen sind zu verschiedenen Meerschweinchenurinen (I—IV) und Menschenharn (V) gesetzt worden.

Nach Brummer (1940) wird die Reaktion Tryptophan-Vanillin-Schwefelsäure durch Nitrate und Nitrite auch in sehr kleinen Konzentrationen gestört. Da Harn diese beiden Gruppen von Verbindungen enthält, liegt die Annahme nahe, dass auch die Reaktion mit Indol von diesen Stoffen gestört wird und dass die für verschiedene Harne wechselnden Konstanten für das Verhältnis zwischen Extinktion und Indolkonzentration gerade durch den wechselnden Nitrat- und Nitritgehalt der Harne bedingt sind.

TABELLE 19.

Einwirkung von Kaliumnitrat und Natriumnitrit auf die Extinktionswerte bei Bestimmung von Indol.

Die Bestimmungen wurden mit den Portionen I und II ausgeführt (siehe das Arbeitsschema), und beide wurden mit 0,5 ccm einer Kaliumnitrat- oder Natriumnitritlösung von wechselnder Konzentration versetzt. Die Bestimmungen wurden in reinen Indollösungen gemacht.

Konzentration der Lösung in %	2	1	0,1	0,05	0,01	0,001	0,0001
Kaliumnitrat E	0,05	0,02	0,01	0,08	0,59		
Natriumnitrit E	0	0	0	0	0,01	0,03	0,46
Kein Zusatz E	0,66						

Wie Tabelle 19 zeigt, wird die Reaktion zwischen Indol und Vanillin-Schwefelsäure auch durch so niedrige Nitrat- und Nitritkonzentrationen gehemmt, wie sie im Harn vorkommen dürften. Eine Beseitigung der Nitrate und Nitrite aus dem Harn dürfte sich kaum durchführen lassen. Bei einer Untersuchung der Verhältnisse bei anderen Aldehyd-Säure-Reaktionen zeigte es sich, dass auch diese gegen Zusätze von Nitraten und Nitriten empfindlich waren. Die einzige Möglichkeit, die Aldehyd-Säure-Reaktion für Indol im Harn quantitativ anzuwenden, scheint dann die zu sein, mit jeder einzelnen Bestimmung auch eine Kalibrierung zu verbinden. Da für das Verfahren das Beersche Gesetz gilt (Abb. 3), kann sich die Kalibrierung darauf beschränken, den Extinktionswert für eine bekannte Menge Indol in jedem untersuchten Harn festzustellen (siehe unten).

Endgültige Methodik.

5 ccm Harn werden in einem Reagenzglas mit 5 ccm dest. Wasser versetzt. In zwei Reagenzgläser werden je 0,5 ccm dieses auf das doppelte Volumen verdünnten Harns gebracht (Portion I und II) und stehengelassen. Der Rest wird mit Chloroform dreimal in der Schüttelmaschine geschüttelt — jedesmal etwa 5 Min. (Nach jedem Schütteln entfernt man das Chloroform am einfachsten in der Weise, dass man es mit Hilfe einer langen, zu einer Spitze ausgezogenen Glasröhre absaugt, die über eine Sammelflasche an eine Wasserstrahlpumpe angeschlossen ist.) Von

dem ausgeschüttelten Harn wird dann 0,5 ccm in ein Reagenzglas gebracht (Portion III).

Portion I wird mit 0,5 ccm dest. Wasser versetzt.

Portion II wird mit 0,5 ccm einer Indolstandardlösung versetzt (siehe unten).

Portion III wird mit 0,5 ccm dest. Wasser versetzt.

Sämtliche Portionen werden dann mit je 0,1 ccm 0,15 % Vanillinlösung und 4 ccm 60 % Schwefelsäure versetzt.

Die Indolstandardlösung wird wie folgt hergestellt: 50 mg Indol werden in 100 ccm Wasser gelöst (= Stammlösung). Diese Lösung hält sich wenigstens 14 Tage. Die Indolstandardlösung für Meerschweinchenharn wird aus 1 ccm Stammlösung bereitet, die mit dest. Wasser auf 20 ccm aufgefüllt wird, die für Menschenharn aus 0,5 ccm Stammlösung, die auf 20 ccm verdünnt wird.

Dann wird mit dem Filter S50 und in den 1 cm-Küvetten des Zeiss-schen Pulfrich-Photometers teils I gegen III (A), teils II gegen III (B) kolorimetriert. In A ist die Extinktion proportional zum Eigengehalt des Harns an Indol, in B zum Eigengehalt des Harns zuzüglich der bekannten zugesetzten Indolmenge. Zieht man den in A gefundenen Extinktionswert von dem in B gefundenen ab, so erhält man die der bekannten Indolmenge entsprechende Extinktion und findet so die für den fraglichen Harn geltende Konstante für das Verhältnis Extinktion—Indolkonzentration, so dass man nun auch den Eigengehalt des Harns an Indol berechnen kann.

Beispiel für die Berechnung.

Meerschweinchenharn. Tagesmenge: 25 ccm. Ablesung A: 0,09. B: 0,29. B—A = 0,20, was also der Ablesungswert der zugesetzten Indolmenge (0,0125 mg) ist. Ein Extinktionswert von 1,0 entspricht dann 0,0625 mg Indol, die Tagesmenge Indol ist also in dem hier vorliegenden Beispiel: $2 \times 25 \times 0{,}09 \times 0{,}0625 = 0{,}28$ mg.

Empfindlichkeit der Methode.

Die Methode ist in der vorliegenden Arbeit zur Bestimmung der Indolausscheidung im Meerschweinchenharn nach Einverleibung von Indol zur Anwendung gebracht worden, und die untere Grenze waren bei der angegebenen Methodik Tagesmengen von etwa 0,05 mg Indol, was vollauf genügen dürfte, um festzustellen, ob bei Indolbelastungsversuchen eine Ausscheidung freien Indols stattfindet (tägliche Dosen von 10—20 mg). Forbes und Neale (1935) geben an, dass sich die unter verschiedenen pathologischen Verhältnissen ausgeschiedenen Indolmengen beim Menschen nur auf 1—2 mg pro Tag belaufen. In vorläufigen Versuchen mit Menschenharn hat es sich gezeigt, dass man, wenn man nur 3 cm-Küvetten

statt 1 cm-Küvetten verwendet, Indolmengen von bloss 1 γ pro ccm bestimmen kann, was einer Tagesmenge von etwa 1,5 mg entspricht. Noch nicht abgeschlossene Versuche lassen mit Bestimmtheit vermuten, dass man durch Veränderungen der Ausgangsmenge Harn sowie der Schwefelsäurekonzentration auch Bestimmungen in Harnen mit noch niedrigeren Indolkonzentrationen ausführen kann.

Die Methode muss sich also für Bestimmungen in Harnen mit demselben Indolgehalt wie den von FORBES und NEALE untersuchten verwenden lassen. Dank ihrer grösseren Genauigkeit und vor allem ihrer Einfachheit, die Reihenbestimmungen in grossem Massstab ermöglicht, dürfte sie sich auch für klinische Untersuchungen gut eignen können.

Experimentelles.

1. *Die Einwirkung von Schwankungen in der Konzentration der Schwefelsäure- und Vanillinlösungen.*

Wie aus Tab. 20 und 21 hervorgeht, spielen kleinere Veränderungen in der Konzentration der Schwefelsäure- und der Vanillinlösungen keine Rolle für die Extinktionswerte. Bei steigender Schwefelsäurekonzentration tritt jedoch in beiden Harnportionen schon bei 65 % Schwefelsäure eine starke Verdunkelung auf. Diese Verdunkelung war bei 75 % Säure so stark, dass keine Ablesung möglich war, weshalb die relativ niedrige Schwefelsäurekonzentration von 60 % gewählt wurde, trotzdem die Farbreaktion bei höheren Konzentrationen stärker ist.

2. *Die Extraktion.*

Wie aus Tab. 22 hervorgeht, ist die Extraktion des Indols schon nach zweimaligem Schütteln mit Chloroform vollständig, und das dreimalige Ausschütteln, wie es in der Methodik vorgeschrieben wird, dürfte also die Gewähr bieten, dass die Extraktion des Indols vollständig ist.

3. *Die Haltbarkeit der Kolorimetriefarbe.*

Wie aus Tabelle 23 hervorgeht ist die Kolorimetriefarbe noch nach 24 Stunden unverändert.

4. *Stört die Anwesenheit von Tryptophan und Indoxyl im Harn die Indolbestimmung?*

Die Tabellen 24 und 25 zeigen, dass selbst bei einem Vielfachen der unter normalen Verhältnissen im Harn vorkommenden Tryptophan- und Indoxylkonzentration keine Steigerung der Indolwerte eintritt. (Eine geringfügige Steigerung des Extinktionswertes zeigt sich erst bei einem 75 mg% entsprechenden Tryptophanzusatz, und noch bei zehnfacher Erhöhung der Indoxylausscheidung werden dieselben Indolwerte gefunden.)

TABELLE 20.

Einwirkung von Änderungen der Schwefelsäurekonzentration bei Bestimmung von Indol.

Bestimmung in gewohnter Weise.

Bei den Bestimmungen im Harn trat in beiden Portionen mit zunehmender Schwefelsäurekonzentration eine starke Verdunkelung auf, die schon bei 65 % Schwefelsäure das Ablesen erschwerte und es bei 75 % Säure unmöglich machte.

Schwefelsäure-konz. in %	50	60	65	70	75
Indollösung E	0,48	0,62	0,60	0,58	0,58
Harn E	0,25	0,25	0,26	0,35	

TABELLE 21.

Einwirkung von Änderungen der Vanillinkonzentration auf die Extinktionswerte bei Bestimmung von Indol.

Bestimmung in gewohnter Weise. (208 mg pro 100 ccm benutzte Brummer (1940) bei seiner Tryptophanbestimmungsmethode.)

Vanillin mg pro 100 ccm	208	145,6	62,4	20,8	10,4
Indollösung E	0,74	0,74	0,73	0,57	0,35
Harn E	0,29	0,30	0,30	0,285	0,25

TABELLE 22.

Die Extraktion mit Chloroform.

Anzahl d. Extraktionen	2	3	4	5
Wässrige Indollösung E	0,61	0,61	0,61	0,58
Menschenharn E	0,56	0,57	0,56	0,56
Meerschweinchenharn E	0,39	0,42	0,41	0,40

TABELLE 23.

Die Haltbarkeit der Kolorimetriefarbe.

Ablesung nach	10 Min.	40 Min.	18,5 Stdn.	24 Stdn.
Harn I E	0,40	0,41	0,415	0,41
Harn II E	0,92	0,925	0,925	0,92

TABELLE 24.

Die Einwirkung eines Zusatzes von Tryptophan auf die Bestimmung von Indol.

Indolgehalt des Harns 0,02 mg pro ½ ccm.

Zugesetztes Tryptophan in mg pro ½ cmm Harn	0	0,125	0,375	0,625
E	0,41	0,41	0,44	0,48

TABELLE 25.

Die Einwirkung des Indoxylgehaltes im Harn auf die Indolbestimmung.

4 Meerschweinchen bekamen täglich wechselnde Mengen Indol eingespritzt. Der Harn wurde in Perioden von 24 Stdn. gesammelt. Die Werte der Indoxyl- und Indolausscheidung sind die Mittelwerte der Bestimmung an sämtlichen Tieren.

Versuchsperiode	1	2	3	4	5
Indolzufuhr mg/Tag	0	10	20	20	20
Indoxylausscheidung mg/Tag	1,70	8,1	15,6	12,6	14,3
Indolausscheidung »	0,11	0,12	0,09	0,19	0,11

5. *Die Zuverlässigkeit der Methodik.*

Da für jede Bestimmung eine Kalibrierung notwendig ist und das Ergebnis somit von zwei Ablesungen abhängt, sind die Fehler bei diesem Verfahren grösser als z. B. bei der obengeschilderten Salizylsäurebestimmungsmethode.

Bei 10 Bestimmungen im selben Harn wurden die Ergebnisse in γ pro ½ ccm Harn (die Menge, in der die Bestimmung gemacht wird) wie folgt gefunden: 11,4 10,9 10,2 10,9 10,5 11,7 11,0 9,3 10,7 9,6.

Der mittlere Fehler des Mediums beträgt ± 0,23, die Streuung ± 0,72.

B. Bestimmung von Indol (und Tryptophan) in gefärbten Lösungen aus Versuchen in vitro.

Die Extraktion mit Chloroform bei dem vorstehend geschilderten Indolbestimmungsverfahren soll verhindern, dass etwa anwesendes Tryptophan zu hohe Werte varanlassen könnte. Bei Untersuchungen über die Umwandlung von Indol durch Askorbinsäure in vitro kann man diese Fehlerquelle ausser Acht lassen. Die Lösungen sind indessen so stark gefärbt, dass die üblichen Bestimmungsmethoden unbrauchbar sind. Ich habe daher ein einfaches Verfahren nach folgendem Prinzip entwickelt: Die Probe wird in zwei Portionen geteilt, von denen die eine mit Vanillinlösung, die andere mit einer gleich grossen Menge dest. Wassers versetzt wird. Anschliessend werden beide Portionen mit derselben Menge Schwefelsäure versetzt. In beiden Portionen wird die Eigenfarbe der Lösung gleichermassen durch die Schwefelsäure beeinflusst, doch nur in der einen tritt die Farbreaktion Indol-Vanillin-Schwefelsäure ein. Der Farbunterschied ist also dem Indolgehalt proportional.

Hohe Askorbinsäurekonzentrationen beeinflussen die Farbreaktion, und im allgemeinen empfiehlt es sich, auch bei dieser Art der Bestimmung gleichzeitig für jede untersuchte Probelösung eine Kalibrierung vorzunehmen.

Methodik.

In drei Reagenzgläschen werden folgende Lösungen gemischt:

I. 1/2 ccm Probelösung + 1/2 ccm Vanillinlösung (0,15 %) + 1/2 ccm Aq. dest. + 4 ccm 60 % Schwefelsäure.

II. 1/2 ccm Probelösung + 1/2 ccm Vanillinlösung + 1/2 ccm Indolstandardlösung (siehe S. 69) + 4 ccm Schwefelsäure.

III. 1/2 ccm Probelösung + 1 ccm Aq. dest. + 4 ccm Schwefelsäure.

Kolorimetrie und Berechnung wie auf S. 69. (Die Bezifferung oben entspricht den Portionen I—III S. 69.)

Die experimentelle Grundlage dieses Verfahrens ist die gleiche wie bei dem vorigen, und es soll hier nur die gute Übereinstimmung zwischen Extinktionswerten und Indolkonzentration aufgezeigt werden (Abb. 4 und 5).

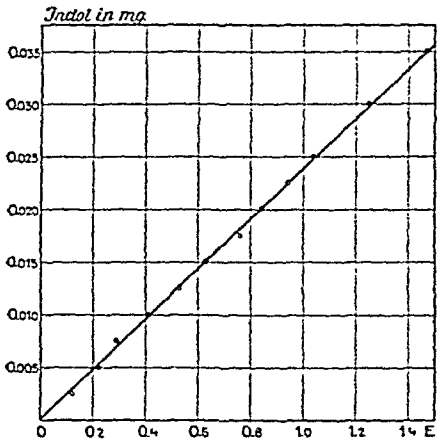

Abb. 4. *Das Verhältnis Indolkonzentration/Extinktionswert.* (Wasserlösung.)

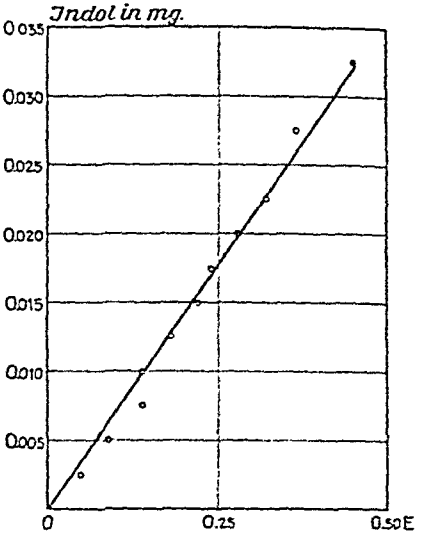

Abb. 5. *Das Verhältnis Indolkonzentration/Extinktionswert.* (Lösung aus In-vitro-Versuch.)

Mittels desselben Verfahrens lässt sich auch Tryptophan bestimmen. Man muss dann jedoch die Indolstandardlösung gegen eine Tryptophanstandardlösung von gleicher Stärke austauschen und das Filter S53 statt S50 benutzen.

IV. Die quantitative Bestimmung von Indoxyl im Harn ist nach Grönwall (1938) ausgeführt worden.

V. Quantitative Bestimmung von Askorbinsäure im Harn.

Das Problem der quantitativen Bestimmung von Askorbinsäure im Harn ist regem Interesse begegnet, das umfangreiche Schrifttum wolle man in den bereits vorliegenden Zusammenstellungen, beispielsweise bei Bessey (1938), Lanke (1938) und in der *Annual Review of Biochemistry* nachschlagen.

Von den zahlreichen vorgeschlagenen Methoden dürften diejenigen, die sich auf die Verwendung von Dichlorphenolindophenol als Reagens (Tillmans'sches Reagens) gründen, wegen ihrer Einfachheit und schnellen Ausführung die übrigen verdrängt

haben. Mit dem TILLMANS'schen Reagens reagieren indessen auch mehrere andere reduzierende Verbindungen, und dies macht sich vor allem dann bemerkbar, wenn das Untersuchungsmaterial mit Schwefelwasserstoff oder einem anderen Reduktionsmittel behandelt worden ist, in der Absicht, auch die Dehydroaskorbinsäure zu bestimmen.

Verbindungen, welche die Askorbinsäurebestimmung stören, sind u. a. Glutathion und Cystein. Ihre Einwirkung lässt sich indessen dadurch beseitigen, dass man in saurem Milieu titriert (BIRCH, HARRIS und RAY 1933). Verwendet man Metaphosphorsäure, so erzielt man damit gleichzeitig einen Oxydationsschutz für die Askorbinsäure, der dem durch andere Säuren bewirkten überlegen ist (FUJITA und IWATAKE 1935). Ein anderer Weg ist von EMMERIE und VAN EEKELEN (1934) angegeben worden, welche die Probelösung mit Merkuriazetat behandeln und dann mittels Schwefelwasserstoff sowohl den Überschuss an Merkuriazetat beseitigen als die Dehydroaskorbinsäure in Askorbinsäure überführen. Auch Thiosulfat kann auf diese Weise unschädlich gemacht werden.

TAUBER und KLEINER (1935) haben eine Modifikation der Titrierung mit dem TILLMANS'schen Reagens angegeben, die gute Möglichkeiten bieten dürfte, korrekte Resultate zu erzielen. Sie titrieren mit dem TILLMANS-Reagens teils in einer unbehandelten Probelösung, teils in einer Probelösung, in der die Askorbinsäure durch Einwirkung eines angeblich spezifisch askorbinsäurezerstörenden Enzyms (Askorbinsäureoxydase, Askorbinase) zerstört worden ist.

Mittels einer solchen Methodik konnten SCARBOROUGH und STEWART (1937) zeigen, dass auch nach Ausfällung mit Merkuriazetat und Ansäuerung noch andere reduzierende Substanzen als Askorbinsäure (oder Glutathion, Cystein und Thiosulfat) im Harn vorhanden sind.

Dasselbe wurde mit einem anderen Verfahren von EVELYN, MALLOY und ROSEN (1938) gezeigt. Mit Hilfe eines photoelektrischen Kolorimeters konnte die Entfärbung des TILLMANS-Reagens nach dem Zusatz der Probelösung in sehr kleinen Zeitabständen verfolgt werden. Es zeigte sich dabei, dass die Askorbinsäure das TILLMANS-Reagens augenblicklich entfärbt, während

andere Verbindungen, die das Reagens reduzieren können, bedeutend langsamer wirken. Es ergab sich, dass Harn auch nach Fällung mit Merkuriazetat noch Verbindungen enthält, die das TILLMANS-Reagens reduzieren, die aber nicht Askorbinsäure sein können.

Diese anderen reduzierenden Verbindungen haben starkes Interesse gefunden, sind aber nach wie vor unbekannt. WIDENBAUER (1936) gibt an, Harnfarbstoffe könnten störend einwirken, und auch andere Verbindungen sind vorgeschlagen worden, z. B. Homogentisinsäure und Polyphenole (BORSOOK et al. 1937).

Am einfachsten dürfte man die Fehlerquellen, die diese Stoffe bei Askorbinsäurebestimmungen in mit Schwefelwasserstoff behandeltem Harn ausmachen, dadurch vermeiden können, dass man ein spezifisch askorbinsäurezerstörendes Enzym verwendet. Doch besteht Grund zu vermuten, dass das bisher angewandte Enzym nicht spezifisch war.

So erhielten LUNDE und LIE (1938) bei einer Untersuchung über den Askorbinsäuregehalt von Algen nach einem Verfahren mit Askorbinsäureoxydase Werte, die sie im Tierversuch nicht bestätigen konnten.

Zu ähnlichen Ergebnissen kamen DIEMAIR, FRESENIUS und ARNOLD (1942) bei Untersuchungen von Dörrgemüse, das im Zusammenhang mit dem Dörren erwärmt worden war.

Später hat RÖNNERSTRAND (1943) zeigen können, dass das Fucosan in Braunalgen durch Gurkensaft oxydiert werden kann. Diese Oxydation vollzieht sich jedoch bedeutend langsamer als die Askorbinsäureoxydation.

Die Behandlung mit Schwefelwasserstoff in der Absicht, auch die Dehydroaskorbinsäure bestimmen zu können, wurde schon von TILLMANS, HIRSCH und DICK (1932) in die ursprüngliche Bestimmungsmethodik eingeführt und findet seitdem allgemeine Verwendung in der experimentellen Arbeit, klinische Askorbinsäurebelastungsversuche ausgenommen.

JOHNSSON und ZILVA (1934) zeigten indessen, dass in grossen Mengen zugeführte Dehydroaskorbinsäure in Form von Askorbinsäure ausgeschieden wird, während sich keine Dehydroaskorbinsäure nachweisen lässt. Der Versuch wurde von BORSOOK et al. (1937) mit demselben Ergebnis wiederholt. Bei allen diesen

Untersuchungen wurde der Harn in Metaphosphorsäure gesammelt, und es liegt die Annahme nahe, dass wenn Dehydroaskorbinsäure im Harn auftritt, dies eine Folge einer während des Sammelns erfolgten Oxydation ist, während dagegen ein verstärktes Reduktionsvermögen nach Behandlung mit Schwefelwasserstoff in Harn, der in Metaphosphorsäure gesammelt worden ist, nicht durch Askorbinsäure bedingt ist.

Die allgemein im Schrifttum vertretene Meinung scheint zu sein, dass man bei direktem Titrieren mit dem TILLMANS-Reagens in einem angesäuerten Harn Werte erhält, die zu praktischen Zwecken als für Askorbinsäure spezifisch angesehen werden können. Behandelt man dagegen Harn mit Schwefelwasserstoff, so nimmt die Menge reduzierender Verbindungen zu, und auf Grund von Untersuchungen mit einem askorbinsäurezerstörenden Enzym führt man diese Steigerung wenigstens zum grösseren Teil auf andere Verbindungen als Askorbinsäure zurück. Im allgemeinen dürfte man jedoch der Ansicht sein, festgestellt zu haben, dass der Harn auch kleine Mengen Dehydroaskorbinsäure enthält. Die Spezifität des Enzyms scheint nicht mit Rücksicht auf die Verwendung bei Harnuntersuchungen geprüft worden zu sein.

Die Untersuchungen von JOHNSSON und ZILWA sowie BORSOOK et al. lassen entschieden vermuten, dass keine Ausscheidung von Dehydroaskorbinsäure stattfindet. Direktes Titrieren mit dem TILLMANS-Reagens in angesäuertem Harn ist so unvergleichlich viel einfacher als ein Verfahren, dass sowohl Behandlung mit Schwefelwasserstoff als die Verwendung eines askorbinsäurezerstörenden Enzyms einschliesst, dass es praktisch von grossem Interesse wäre zu untersuchen, ob man Rücksicht auf eine etwaige Ausscheidung von Dehydroaskorbinsäure im Harn nehmen muss.

Der natürliche Gang einer solchen Untersuchung wäre dann wohl folgender:

1. zu untersuchen, ob man den Harn sammeln kann, ohne dass während des Sammelns Askorbinsäure zu Dehydroaskorbinsäure oxydiert wird,

2. festzustellen, ob die Askorbinase bei Verwendung im Harn spezifisch askorbinsäurezerstörend wirkt,

3. mit Hilfe dieses kontrollierten Enzyms zu untersuchen, ob Dehydroaskorbinsäure ausgeschieden wird oder ob die Verstärkung der reduzierenden Eigenschaften des Harns nach Behandlung mit Schwefelwasserstoff irgendeiner anderen Verbindung zuzuschreiben ist, sowie

4. zu untersuchen, um welche Verbindung es sich dabei gegebenenfalls handelt.

Experimentelles.

Die Askorbinsäurebestimmung mit oder ohne vorherige Behandlung mit Schwefelwasserstoff wurde mittels folgender Methoden vorgenommen:

1. Titration mit dem TILLMANS-Reagens in einer mit Metaphosphorsäure angesäuerten Lösung: Bestimmung nach TILLMANS.

2. Wie 1., doch mit Verwendung eines askorbinsäurezerstörenden Enzyms nach TAUBER und KLEINER (1935): Bestimmung nach TAUBER. Als Enzymmaterial wurde ausgepresster Gurkensaft (Cucumis flexuosus) verwendet.

Am einfachsten lässt sich dieser aufbewahren, wenn man ihn zu Würfeln gefrieren lässt. Noch nach 7 Monaten liefern diese beim Auftauen einwandfreies Enzymmaterial. Verwahrt man den Gurkensaft dagegen in flüssigem Zustand in einem Glasgefäss, so tritt sehr bald (nach einem Tage) ein thermostabiler askorbinsäurezerstörender Faktor auf, über dessen Spezifität sich selbstverständlich nichts aussagen lässt.

3. Kolorimetrische Bestimmung nach FUJITA und EBIHARA (1937) mit Phospho-18-wolframsäure als Reagens: Bestimmung nach FUJITA.

Bei Behandlung mit Schwefelwasserstoff liess ich diesen die Probelösung, welche mit Metaphosphorsäure versetzt worden war, so dass die Konzentration etwa 10 % betrug, 1 Stunde lang durchströmen, worauf die Lösung unter Verschluss bis zum folgenden Tage stehengelassen wurde. Der Schwefelwasserstoff wurde durch Hindurchleiten von Stickstoff während 2 $^1/_2$ Stunden entfernt. Schon nach 1 $^1/_2$ Stunden wurden konstante Titrationswerte erhalten, und selbst nach 5stündigem Hindurchleiten des Stickstoffs waren keine weiteren Veränderungen mehr zu beobachten.

Der Stickstoffstrom muss ziemlich kräftig sein, um den Schwefelwasserstoff schnell beseitigen zu können, und in gewissen Harnen sowie vor allem in pflanzlichem Material kommt es oft zu einer lästigen Schaumbildung, die grosse Verluste an Probelösung verursachen kann. Mittels eines »Schaumfängers», dessen Aussehen aus Abb. 6 hervorgeht, lässt sich diesem Übelstand abhelfen. Der »Schaumfänger» braucht nicht genau in die Reagensglasmündung zu passen und besteht am einfachsten aus einem umgekehrten Erlenmeyerkolben mit einer Öffnung im Boden.

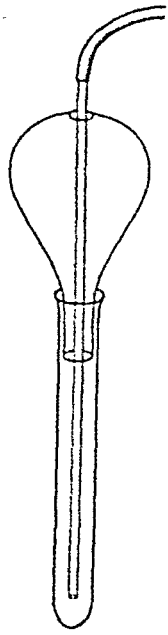

Abb. 6. »*Schaumfänger*».

1. *Das Sammeln des Harns.*

Sowohl bei klinischen Untersuchungen als bei Tierversuchen verfährt man in bezug auf Sammeln und Aufbewahren des Harns oft so, dass man ihn mit einer Toluolschicht überdeckt, um dem Luftzutritt und Bakterienwachstum vorzubeugen. Im allgemeinen hat man dieses Verfahren mit Schwefelwasserstoffbehandlung des Harns verbunden, um teils etwaige durch Oxydation beim Sammeln entstandene Verluste zu kompensieren und teils die Bestimmung eventuell ausgeschiedener Dehydroaskorbinsäure zu ermöglichen.

Wie aus Tabelle 26 hervorgeht, vollzieht sich aber in Harn, der nur durch Toluol geschützt ist, sehr bald eine fast vollständige Oxydation, die grösstenteils irreversibel ist.

Versetzt man dagegen den Harn mit Metaphosphorsäure und Toluol, so ist der Askorbinsäuregehalt noch nach 24 Stunden unverändert (Tabelle 27).

TABELLE 26.

Der Harn muss in Metaphosphorsäure gesammelt werden, damit keine irreversible Oxydation der Askorbinsäure stattfindet.

5 Meerschweinchen bekamen täglich 100 mg Askorbinsäure eingespritzt. Askorbinsäurebestimmungen nach Tillmans. Metaphosphorsäurezusatz 10 ccm 20 % Lösung. Toluolzusatz etwa 15 ccm.

Tier Nr.		1	2	3	4	5
Versuchstag	Kolben mit:	C-Vitaminausscheidung in mg/Tag. Unterstrichene Werte: Bestimmung nach H_2S-Behandlung				
1	Metaphosphorsäure + Toluol	27,5	28,4	30,5	43,8	33,4
2	Toluol	2,2 5,5	11,4 17,1	5,5 9,9	15,8 19,1	13,7 18,3
3	Metaphosphorsäure + Toluol	47,5	22,5	36,3	37,5	42,4

TABELLE 27.

Harn, der im Laufe von 24 Stunden in Kolben gesammelt worden ist, die 15 ccm 20 % Metaphosphorsäure und 15 ccm Toluol enthalten, zeigt nach weiteren 24 Stunden praktisch unveränderte Askorbinsäurewerte.

Meerschweinchen Nr.	1	2	3	4	5	6	7	8	9	10
cc Tillmans	0,85	0,85	0,95	0,50	0,20	0,85	1,0	0,40	0,50	0,50
Nach 4 Stdn	0,85	0,85	1,0	0,45	0,20	0,80	1,0	0,35	0,50	0,50
Nach 24 Stdn	0,85	0,85	1,0	0,45	0,20	0,75	0,90	0,35	0,50	0,50

2. *Die Spezifität der Askorbinase bei Bestimmung von Askorbinsäure im Harn nach* TAUBER.

Man dürfte annehmen können, dass der Harn skorbutischer Meerschweinchen keine Askorbinsäure, im übrigen aber die Bestandteile des normalen Harns enthält. Ist das verwendete Enzymmaterial spezifisch auf Zerstörung der Askorbinsäure angestellt, so muss man bei Bestimmungen in skorbutischem Harn nach TAUBER eine Askorbinsäureausscheidung gleich null auch nach Behandlung mit Schwefelwasserstoff finden. Wie aus Tabelle 28 ersichtlich, war dies auch der Fall. Ausserdem wurden Bestimmungen nach TILLMANS und nach FUJITA in denselben

TABELLE 28.

Vergleichende Bestimmungen der Ausscheidung reduzierender Verbindungen im Harn mittels der Methoden von Fujita, Tillmans und Tauber, in sämtlichen Fällen nach Behandlung des Harns mit Schwefelwasserstoff.

Der Versuch umfasst 5 Meerschweinchen, die Menge der reduzierenden Verbindungen ist in mg Askorbinsäure pro Tag und Tier ausgedrückt (Mittelwerte für sämtliche Tiere).

Tage nach Beginn des Skorbutversuchs			3	6	9	12	18	24	27
Vitamin-C-Zufuhr	Überschuss		0..	....	....	....	....	....	..0
Vitamin-C-Ausscheidung Askorbinsäurebestimmung nach:									
Fujita mg/Tag	2,2	2,8	2,4	2,1	2,2	1,5			
Tillmans »	7,7	7,3	3,4	3,3	3,4		4,0	2,6	4,8
Tauber »	0,2	0,2	0,2	0,2	0	0	0	0	0

Harnen und ebenfalls nach vorheriger Behandlung mit Schwefelwasserstoff gemacht. Diese beiden Methoden lieferten indessen Ergebnisse, die besagen würden, dass mit fortschreitendem Skorbut eine fortgesetzte oder gar ansteigende Askorbinsäureausscheidung stattfinden würde.

3. *Wird Dehydroaskorbinsäure im Harn ausgeschieden?*

Um festzustellen, ob mit dem Harn Dehydroaskorbinsäure ausgeschieden wird, wurde folgender Versuch gemacht:

Meerschweinchen bekamen wechselnde Mengen Askorbinsäure eingespritzt. Der Harn der Tiere wurde 24 Stunden lang in Kolben gesammelt, die 15 ccm 20 % Metaphosphorsäure und eine etwa cm-dicke Toluolschicht enthielten. Unter diesen Verhältnissen tritt keine Oxydation ausgeschiedener Askorbinsäure ein (siehe Tabelle 27). Die Askorbinsäure wurde nach TILLMANS und nach TAUBER mit und ohne Schwefelwasserstoffbehandlung bestimmt.

Dabei ergab sich, wie aus Tabelle 29 hervorgeht, dass man, wenn die Bestimmung in 1 ccm Harn gemacht wird (der also etwa zu gleichen Teilen aus Meerschweinchenharn und Metaphosphorsäure besteht), bei Bestimmung nach TILLMANS mit und ohne Schwefelwasserstoffbehandlung dasselbe Resultat erhielt. Falls wegen geringen Askorbinsäuregehaltes in einer Harnmenge von nicht weniger als 5 ccm titriert werden musste, so ergab sich dagegen bei Bestimmung nach TILLMANS eine erheblich ge-

TABELLE 29.

Die Zunahme des Reduktionswertes im Harn nach Behandlung mit Schwefelwasserstoff ist nicht durch Dehydroaskorbinsäure bedingt.

Die Werte der Tabelle sind die Mittelwerte aus Bestimmungen in verschiedenen Meerschweinchenharnen und geben den Verbrauch an 0,1 % Dichlorphenolindophenol an.

Vitamin-C Zufuhr	100 mg/Tag	20 mg/Tag		
Bestimmung in:	1 ccm Harn	5 ccm Harn		
Methode:	Tillmans	Tillmans	Tauber	Tauber Restreduktion
Vor H_2S-Behandlung	1,28	0,34	0,22	0,12
Nach H_2S-Behandlung	1,30	0,58	0,18	0,40
Anzahl d. Harnproben	50	12	12	12

steigerte Reduktionsfähigkeit des Harns, wenn dieser vorher mit Schwefelwasserstoff behandelt worden war.

Bei Bestimmung nach TAUBER zeigte es sich indessen, dass die Askorbinsäure eher etwas geringer war. Die erhöhte Reduktionsfähigkeit war also durch reduzierende Verbindungen bedingt, die von dem Enzym nicht angegriffen wurden und keine Askorbinsäure sein können. Diese liefern den sog. Restreduktionswert (d. h. den Titrierwert bei Bestimmung nach TAUBER, den man nach Zusatz von Enzym findet).

Der Versuch zeigt also, dass keine Dehydroaskorbinsäure ausgeschieden wird und dass die Behandlung mit Schwefelwasserstoff überflüssig ist.

Aus der obengenannten Tabelle (Tabelle 29) geht indessen hervor, dass man auch in nicht mit Schwefelwasserstoff behandeltem Harn einen geringfügigen Restreduktionswert nach TAUBER erhält (einer Askorbinsäureausscheidung von etwa 0,36 mg/Tag bei Bestimmung in 5 ccm Probe entsprechend). Zwar besagt dies, dass Dichlorindophenol nicht einmal im nicht mit Schwefelwasserstoff behandelten Harn ein ganz spezifisches Askorbinsäurereagens ist, doch ist der Fehler so klein, dass er keine Rolle spielt, wenn in 1 ccm Harn titriert werden kann. (Er würde in normalem Harn nur 0,02 ccm der verwendeten 0,1 % Reagenzlösung entsprechen.)

TABELLE 30.

Der Zusammenhang zwischen Urochrom A und Restreduktion.

In Meerschweinchenharn wurde teils das Urochrom A, teils die Askorbinsäure gemäss Fujita, Tillmans und Tauber nach Behandlung mit Schwefelwasserstoff bestimmt. Die Ergebnisse nach Fujita und Tillmans wurden um die Ergebnisse nach Tauber (d. h. um den korrekten Askorbinsäurewert) vermindert, und auf diese Weise erhielt man einen Ausdruck für die Restreduktion (die durch andere Verbindungen als Askorbinsäure bedingte Reduktion). Die Harne wurden nach ihrem Urochrom-A-Gehalt gruppiert, und für jede Gruppe wird das Mittel aus den entsprechenden Restreduktionswerten angegeben.

Urochrom-A. E	0,00–0,19	0,20–0,29	0,30–0,39	0,40–0,49	0,50–0,59	0,60–0,69	0,70–0,79	0,80–
Restreduktion in mg Askorbinsäure/Tag mit:								
Fujita	2,11	2,3	2,5	3,21	2,96	4,98	3,58	8,36
Tillmans	5,24	3,88	4,47	4,42	7,10	8,81	10,4	15,3
Anzahl Harnproben	3	11	22	19	9	10	6	11

4. *Das Urochrom A verursacht nach der Behandlung des Harns mit Schwefelwasserstoff diejenige Reduktion des Tillmans-Reagens, die nicht durch Askorbinsäure bedingt ist.*

In Meerschweinchenharnen wurde sowohl die Askorbinsäureausscheidung nach Tillmans, Fujita und Tauber nach vorheriger Behandlung mit Schwefelwasserstoff als auch die Urochrom-A-Ausscheidung nach Ekman bestimmt. Die Askorbinsäurewerte nach Tillmans und Fujita wurden um die korrekten Askorbinsäurewerte nach Tauber vermindert. Man erhielt so einen Ausdruck für den nicht durch die Askorbinsäure bedingten Anteil der Reduktion = die Restreduktion. Die Ergebnisse aus diesen Versuchen sind in Tabelle 30 zusammengefasst, wo die Harne je nach den gefundenen Urochrom-A-Werten gegliedert sind, für jede »Urochrom-A-Gruppe» ist das Mittel der Restreduktionswerte nach Tillmans und Fujita sowie die Anzahl der untersuchten Harne angegeben. Namentlich die nach Tillmans gewonnenen Restreduktionswerte und die Urochrom-A-Werte stimmen quantitativ sehr gut miteinander überein, und eine gleichsinnige Tendenz macht sich auch betreffs der nach Fujita gefundenen Restreduktionswerte bemerkbar.

Betreffs des Tillmans'schen Reagens ist die Untersuchung in der Weise fortgeführt worden, dass Urochrom A isoliert und nach Zusatz von Metaphosphorsäure mit Schwefelwasserstoff behandelt wurde. Es ergab

sich, dass diese Urochrom-A-Lösung das TILLMANS-Reagens zu reduzieren vermochte. Der Versuch ist mit sämtlichen in Kap. VII behandelten Urochrom-A-Präparaten angestellt worden.

Besprechung der Ergebnisse.

Die Versuchsergebnisse dürften sich folgendermassen zusammenfassen lassen:

1. Bei Askorbinsäureuntersuchungen im Harn muss der Harn in Metaphosphorsäure und unter Toluol gesammelt werden, da sonst eine baldige irreversible Oxydation der Askorbinsäure eintritt.

2. Der Extrakt aus Schlangengurken enthält eine Askorbinase, die keinen anderen Harnbestandteil als Askorbinsäure angreift.

3. Die Askorbinsäure wird nur in nicht oxydierter Form ausgeschieden, weshalb sich die Behandlung mit Schwefelwasserstoff erübrigt. Bei Bestimmung in 1 ccm Harn (in der gleichen Menge Metaphosphorsäure gesammelt) kann Dichlorphenolindophenol (0,1 %) als ein ausreichend spezifisches Askorbinsäurereagens gelten.

4. Behandelt man Harn mit Schwefelwasserstoff, so wird nicht nur Askorbinsäure, sondern auch Urochrom A reduziert, und dieses Urochrom A kann das TILLMANS-Reagens reduzieren.

Im Zusammenhang mit der Feststellung, dass es wahrscheinlich das Urochrom A ist, welches nach Schwefelwasserstoffbehandlung des Harns zu hohe »Askorbinsäurewerte» liefert, ist eine Untersuchung von WÖRDERHOFF (1940) von Interesse, in der gezeigt wird, dass die Restreduktion bei Bestimmungen von Askorbinsäure im Serum nach TAUBER bei Patienten ansteigt, bei denen auf Grund niedriger Serum-Askorbinsäurewerte ein C-Vitaminmangel angenommen werden kann. Die Analogie mit der von EKMAN (1940) beobachteten Steigerung der Urochrom-A-Werte im Harn bei C-Vitaminmangel erscheint eindeutig, und man darf wohl annehmen, dass auch die von WÖRDERHOFF bei C-Vitaminmangel beobachtete erhöhte Restreduktion im Serum auf das Urochrom A zurückzuführen ist. Es erscheint ferner möglich, dass die auf S. 37 erwähnte Beobachtung von DANIELS

und EVERSON, dass die Askorbinsäureausscheidung bei Kindern nach Zufuhr von Azetylsalizylsäure anstiege, so zu erklären wäre, dass eine erhöhte Urochrom-A-Ausscheidung die wahre Ursache der gesteigerten »Askorbinsäurewerte» gewesen ist.

VI. Die quantitative Bestimmung von Histidin ist nach Niendorf (1939) ausgeführt worden.

VII. Quantitative Bestimmung des Phenols.

Zur Bestimmung des Phenols bediente ich mich eines kolorimetrischen Verfahrens mit p-nitranilin als Phenolreagens nach BÖHM und GRÜNER (1936). Um störende Farbstoffe zu beseitigen, schüttelte ich die Probelösungen (Harn oder Lösungen von In-vitro-Versuchen) mit Permutit, wie es MARENZI und BANFI (1936) vorgeschlagen haben. Um auch gebundenes Phenol bestimmen zu können, habe ich die Phenolbestimmungen nach Hydrolyse der Probierlösung mit HCl im kochenden Wasserbad nach THEIS und BENEDICTS (1924) Anweisungen für die Bestimmung des Gesamtphenols im Harn ausgeführt.

VIII. Bestimmung von Polyphenolen.

Im Verlauf der Untersuchungen zeigte es sich, dass die Umwandlung aromatischer Verbindungen durch Askorbinsäure als eine Oxydation aufgefasst werden muss. Damit ergab sich der Wunsch, diesen Oxydationsablauf quantitativ zu verfolgen. Es durfte angenommen werden, dass sich dabei Stoffe von Polyphenolnatur, also Dioxy- und Trioxybenzole bilden würden. Diese Stoffe ergeben eine Farbreaktion mit Phosphormolybdensäure, die BRIGGS (1922) zur Bestimmung von Homogentisinsäure verwertet hat. BRIGGS vergleicht die von Homogentisinsäure mit Phosphormolybdensäure gebildete Farbe mit derjenigen Farbe, die eine Hydrochinonstandardlösung mit demselben Reagens liefert. Das Verfahren lässt sich also ebensogut zur Bestimmung von Hydrochinon verwenden. Eiweissstoffe trüben die Lösungen und Sulfide ergeben mit dem Reagens eine ähnliche Farbe wie

die, welche z. B. mit Hydrochinon erhalten wird. Um diese Fehlerquellen zu vermeiden, behandelt BRIGGS die Probelösung zunächst mit Trichloressigsäure und Silbersulfat.

Man hat feststellen können, dass die meisten Polyphenole, die aus dem Harn isoliert worden sind, an Schwefelsäure oder in gewissen Fällen an Glykuronsäure gebunden waren (NEUBAUER-HUPPERT 1913). Ich habe eine Reihe vergleichender Polyphenolbestimmungen nach BRIGGS in verschiedenen Harnen mit und ohne vorherige Salzsäurehydrolyse der Probelösungen gemacht, und es zeigte sich dabei auch, dass nach der Hydrolyse höhere Werte herauskamen.

Das ursprüngliche BRIGGS'sche Verfahren wurde daher mit einer Hydrolyse verbunden, und die Polyphenolbestimmungen wurden nach folgendem Arbeitsschema ausgeführt:

3 ccm Harn werden im Reagenzglas mit 3 ccm 10 % Trichloressigsäure und 3 ccm 0,5 % Silbersulfat zusammengebracht. Nachdem das Reagenzglas geschüttelt worden ist, lässt man es mindestens 10 Min. stehen. Dann wird 1 ccm 25 % Natriumchlorid beigegeben und nach vorherigem Schütteln filtriert. 5 ccm des Filtrats werden mit 10 ccm Aq. dest. und 1 ccm 10-n Salzsäure versetzt und 15 Min. in ein kochendes Wasserbad gestellt. Nach Abkühlung auf Zimmertemperatur werden 2 ccm 1 % primäres Kaliumphosphat und 2 ccm 5 % Ammoniummolybdat in 5-n Schwefelsäure zugesetzt. Nach genau 5 Min. liest man die Extinktion im ZEISS-PULFRICH-Photometer mit dem Filter S72 in 3 cm-Küvetten mit dest. Wasser als Vergleichslösung ab. (Bei Verwendung von kupferhaltiger Salzsäure zur Hydrolyse [siehe unten] wird dies jedesmal angegeben.)

Angabe der Resultate.

Verschiedene Polyphenole ergeben je nach ihrer Konstitution eine verschieden starke Farbreaktion mit Phosphormolybdensäure und in gewissem Grade unterschiedliche Farbnüancen. Es ist daher nicht möglich, bei Verwertung der Reaktion z. B. in Harn die gefundenen Extinktionswerte in eine gewisse Menge Polyphenole zu verwandeln, sondern man kann nur relative Werte erhalten.

Das Ergebnis wird daher nur als der Extinktionswert bei Bestimmung nach dem oben angegebenen Reaktionsschema in einer bestimmten Tagesmenge Harn angegeben (E-BRIGGS).

Kontrollversuche.

Sowohl die Dioxyphenole Brenzkatechin und Hydrochinon als das Trioxyphenol Brenzgallol ergeben mit dem angewandten Filter eine maximale Extinktion.

Bei Doppelbestimmungen erhält man gute Übereinstimmung zwischen den Werten im selben Harn. Die Abweichungen betragen im allgemeinen höchstens 1—2 %.

Unter den speziellen Verhältnissen, unter denen das Verfahren hier zur Anwendung gelangen soll, kommen grosse Schwankungen im Askorbinsäure- und Urochrom-A-Gehalt des Harns vor, weshalb die Möglichkeit einer Einwirkung seitens dieser beiden Verbindungen auf die Bestimmung untersucht worden ist.

Askorbinsäure ergibt auch in kleinen Mengen mit Phosphormolybdensäure dieselbe Reaktion wie die Polyphenole. Da der Harn für diese Untersuchungen ohne Zusatz von Metaphosphorsäure gesammelt worden ist, war im allgemeinen alle Askorbinsäure schon während des Sammelns oxydiert worden, die Reste wurden dann beim Kochen mit Salzsäure dekomponiert. In gewissen Fällen war aber trotzdem noch Askorbinsäure zurückgeblieben, und es wurde dann so verfahren, dass die bei der Hydrolyse benutzte Salzsäure mit kleinen Mengen Kupfer (1 % Kupferazetat in der 10-n Salzsäure) versetzt wurde, wodurch die restlose Zerstörung der Askorbinsäure erzielt wurde. Dieses Verfahren bringt jedoch auch kleinere Verluste an Polyphenol mit sich.

Sämtliche isolierten Urochrom-A-Präparate (siehe Kap. VII) reagieren mit Phosphormolybdensäure. In Kontrollversuchen zeigte es sich aber, dass das meiste Urochrom durch den Silbersulfatzusatz gefällt wird, weshalb die Einwirkung von Urochrom A keine nennenswerten Fehler zu verursachen braucht.

Zusammenfassend dürfte man von der Methode sagen können, dass sie starke Mängel aufweist: die Vergleichbarkeit der gefundenen Werte kann dadurch gestört werden, dass verschiedene Polyphenole verschieden starke Farbreaktionen mit Phosphormolybdensäure ergeben, und durch Einwirkung von Urochrom A und Askorbinsäure können zu hohe Werte herauskommen.

Die Methode ist daher nur unter bestimmten kontrollierten Verhältnissen und mit speziellen Versuchsvorrichtungen brauchbar. Der Wert der gefundenen Ergebnisse wird in Einsicht über die Mängel der Methode im Zusammenhang mit den einzelnen Versuchen erörtert werden.

IX. Quantitative Bestimmung von Stoffen, die eine primäre Aminogruppe enthalten.

Stoffe, die eine primäre Aminogruppe enthalten, können nach Diazotierung dieser Aminogruppe mit einem aromatischen Amin gepaart werden und mit diesem einen Diazofarbstoff liefern, der sich kolorimetrisch bestimmen lässt. Auf dieses Prinzip hat MARSCHALL eine quantitative Bestimmung des Sulfanilamids gegründet, die dann von HECHT verbessert und schliesslich auch an anderen Sulfanilsäureamiden von SIMESEN (1939) nachgeprüft worden ist. In einer späteren Arbeit (1940 a) hat SIMESEN das Hydrolysenverfahren bei Bestimmung des im Organismus gepaarten Teiles der Sulfanilsäureamide vereinfacht.

In der vorliegenden Arbeit sind Sulfanilamid und Paraaminobenzoesäure nach dem von SIMESEN angegebenen Verfahren bestimmt worden.

Kontrollversuche.

Wie SIMESEN hervorhebt, muss alles Nitrit, das nach der Diazotierung im Überschuss vorhanden ist, vor Zusatz des aromatischen Amins entfernt werden, da man sonst unrichtige Werte erhält. Zur Beseitigung des Nitrits im Überschuss habe ich Harnstoff verwendet, doch muss, wenn man ein korrektes Resultat erzielen will, die diazotierte Lösung dann vor dem Zusatz des aromatischen Amins mindestens 1/2 Stunde mit einer 2 % Harnstofflösung stehen.

Die Hydrolyse der gepaarten Verbindung wird mit den von SIMESEN angegebenen Verfahren vollständig.

Sulfanilamid, das dem Harn zugesetzt wird, wird quantitativ wiedergefunden.

Die Kolorimetriefarbe hält sich 24 Stunden, vorausgesetzt dass die (alkoholische) Lösung vor Verdunstung geschützt wird.

X. Versuchsvorrichtungen.

A. In vitro.

Die Technik fusst auf EDLBACHERS (1937) Versuchen, Histidin durch Askorbinsäure abzubauen, wobei der Abbau bei Sauerstoffzufuhr in Anwesenheit von dreiwertigem Eisen stattfand.

Die Versuche in vitro sind auf zweierlei Weise ausgeführt worden, und zwar teils nach einer Technik, die sich eng an die EDLBACHERsche anlehnt und die ich im folgenden als Kolbenversuche bezeichne, teils in einer etwas abgewandelten Form, die hier als Schalenversuche bezeichnet wird.

1. Kolbenversuche.

Hier wurden 55 ccm fassende Fraktionskolben benutzt, die an eine Wasserstrahlpumpe angeschlossen waren und durch ein Kapillarrohr, das nahe der Bodenfläche des Kolbens mündete, belüftet wurden. Als Puffer diente prim./sek. Phosphat in einer Gesamtmenge von 25 ccm. Das Gesamtvolumen im Versuch war stets 50 ccm. Um Fällung von Eisenverbindungen zu verhüten, arbeitete ich mit Ferrizitrat statt des von EDLBACHER verwendeten Ferrisulfats. Die Fraktionskolben wurden in ein Wasserbad gebracht, das eine konstante Temperatur von 42° C gewährleistete; die Versuchsdauer betrug im allgemeinen 16—24 Stunden. Bei Zusatz saurer oder basischer Verbindungen wurden diese stets mit Natriumhydroxyd bzw. mit Salzsäure auf das pH der Pufferlösung gebracht.

Die Mengen der Askorbinsäure und zyklischen Verbindungen, in mg ausgedrückt, sind gewöhnlich Teile oder Vielfache des Molekulargewichts der betreffenden Stoffe.

Diejenigen Versuche in vitro, die auf Isolierung des gebildeten Farbstoffs abzielten, sind stets als Kolbenversuche durchgeführt worden, doch in grösserem Massstab und mit Fraktionskolben, die 500 oder 1000 ccm fassten. (Beispiel: $1{,}_{72}$ g Sulfanilamid, $4{,}_{4}$ g Askorbinsäure, 136 mg Ferrizitrat, 500 ccm Pufferlösung pH $5{,}_{8}$, Versuchszeit 48 Stdn.)

2. Schalenversuche.

Bei den Schalenversuchen wurden die Versuchslösungen nicht in der für die Kolbenversuche beschriebenen Art belüftet, sondern das Reaktionsgemisch wurde in flache Glasschalen gegossen, die etwa 20 ccm fassten und die dann bei konstant 42° C im Thermostat abgestellt wurden. Das Gesamtvolumen der Ver-

suchslösungen war ungefähr 15 ccm, die Pufferlösung 10 ccm. Die Sauerstoffzufuhr erfolgte bei diesen Versuchen also nur durch die Berührung der im Verhältnis zum Volumen grossen Flüssigkeitsoberfläche mit der Luft. Nach Beendigung eines Schalenversuchs wurde die Reaktion immer dadurch zum Aufhören gebracht, dass die Lösung mit 5 ccm 20 % Metaphosphorsäure angesäuert wurde.

Auch in den Schalenversuchen kann man eine Farbstoffbildung beobachten, doch ist diese so schwach, dass die Urochrom-A-Methodik nicht brauchbar war. Da es wünschenswert erschien, eine Vorstellung von der Farbstoffbildung zu bekommen, wurden die Versuchslösungen statt dessen direkt kolorimetriert, und zwar mit dem Filter S47 in 3-cm-Küvetten des PULPHRICH-Photometers und mit dest. Wasser als Vergleichslösung. Der gefundene Extinktionswert (im folgenden als »Extinktionswert der Totalfarbstoffbildung» bezeichnet) ist u. a. durch Urochrom A, aber auch durch nicht mit Kupferazetat fällbare Farbstoffe bedingt. Durch Kolorimetrieren in angesäuerter Lösung kann man indessen verhindern, dass Farbe, die durch die Reaktion zwischen Askorbinsäure und dem zugesetzten Eisen bedingt ist, zurückbleibt.

Die Versuche in vitro sind allgemein als Kolbenversuche ausgeführt worden, in gewissen Fällen aber waren Schalenversuche zweckmässiger, nämlich teils wenn mit grossen Versuchsreihen gearbeitet werden musste, teils wenn es sich um sehr schwache Versuchslösungen handelte. Im letzteren Falle durfte nämlich angenommen werden, dass Schwankungen in der Belüftung allzu starke Uneinheitlichkeit der Versuchsergebnisse zur Folge haben könnten.

B. In vivo.

Versuche mit Vitamin C in vivo müssen in den meisten Fällen an Tieren vorgenommen werden, die bei mangelhafter C-Vitaminzufuhr Skorbut bekommen. Bei den vorliegenden Untersuchungen haben also Meerschweinchen als Versuchstiere gedient, während die nicht als wirkliche Skorbutversuche geplanten in gewissen Fällen am Menschen gemacht worden sind.

Vorläufige Untersuchungen über den Zusammenhang zwischen Brunstzyklus und Ausscheidung von Histidin und Indoxyl haben

solche Befunde ergeben, dass ich es für richtig hielt, in den Meerschweinchenversuchen nur mit Männchen zu arbeiten.[1]

Die Tiere wurden in Stoffwechselkäfigen vom gewohnten Typus gehalten, und der Harn wurde in Kolben gesammelt, die zwecks Vermeidung von Bakterienwachstum und Luftzutritt mit einer etwa cm-dicken Schicht Toluol beschickt waren. Bei Bestimmung der Askorbinsäure im Harn sind besondere Massregeln getroffen worden (siehe S. 84).

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[1] 12 Rattenweibchen bekamen täglich 20 mg Askorbinsäure eingespritzt. Ihr Zyklus wurde 3 Wochen lang durch tägliche Vaginalabstriche beobachtet. Der Harn wurde in Zweitageperioden mit einem freien Zwischentag abwechselnd unter Toluol gesammelt. Bestimmt wurde die Indoxyl- und Histidinausscheidung.

Nach den Vaginalabstrichen, die in gewohnter Weise beurteilt wurden, sind in Tabelle 31 die Harnsammelperioden in 4 Gruppen gegliedert: 1) der Harn war unmittelbar vor einer Brunstperiode gesammelt, 2) während einer Brunstperiode, 3) unmittelbar nach einer Brunstperiode und 4) in einer Zwischenperiode. Wie aus der Tabelle hervorgeht, herrscht ein unverkennbarer Zusammenhang zwischen der Indoxyl- und Histidinausscheidung im Harn und dem Brunstzyklus. Beide Stoffe werden während und unmittelbar nach der Brunstperiode stärker ausgeschieden, während der Zwischenperiode und unmittelbar vor einer Brunstperiode ist die Ausscheidung geringer.

Die Untersuchungen werden in anderem Zusammenhang fortgesetzt werden.

TABELLE 31.

Der Brunstzyklus und die Ausscheidung von Histidin und Indoxyl bei der Ratte.

Versuchseinrichtung siehe im Text!

Aufsammlung des Harns	Unmittelbar vor einer Brunstperiode	Während einer Brunstperiode	Unmittelbar nach einer Brunstperiode	In einer Zwischenperiode	Anzahl Bestimmungen
Ausscheidung in mg/Tag von Histidin	0,69	0,74	0,79	0,61	67
Indoxyl	1,12	1,16	1,60	1,33	71

Die Kost war in sämtlichen Tierversuchen die von HAMMARSTEN (1937) angegebene Kost Nr. 211.[1] Diese soll eine Normalkost sein und enthält daher auch das C-vitaminhaltige Hagebuttenmehl. Dieses ist hier aber ausgelassen worden, die C-Vitaminzufuhr wird in jedem einzelnen Versuch besonders angegeben.

Die Kost war eigentlich für Ratten ausgearbeitet worden, hat sich aber auch für Meerschweinchenversuche als geeignet erwiesen: bei genügender Zufuhr von Vitamin C wuchsen die Tiere normal und waren in guter Verfassung, ohne Darreichung von Vitamin C gingen sie in der normalen Zeit an Skorbut zugrunde.

In der vorliegenden Arbeit, die auch Studien über die Ausscheidung eines Farbstoffs, des Urochroms A, im Harn umfasst, dürfte ein wesentlicher Vorzug dieser Kost darin liegen, dass sie keine gefärbten Bestandteile enthält.

Die Tiere bekamen täglich 20—25 ccm Wasser.

In diesem Kapitel werden neue Methoden zur Bestimmung von Urochrom A, Salizylsäure (freier und gebundener) und Indol vorgelegt.

Die Bestimmungsmethoden für Askorbinsäure im Harn sind geprüft worden, wobei folgende Resultate erhalten wurden:

1. Dehydroaskorbinsäure wird im Harn von Meerschweinchen nicht ausgeschieden.

2. Sammelt man den Harn in Metaphosphorsäure und unter Toluol, tritt keine Zerstörung der Askorbinsäure ein, und in den meisten Fällen ist eine direkte Titration mit Dichlorphenolindophenol genügend spezifisch. Titriert man in grösseren Harnvolumina, so bildet das Urochrom A eine ernste Fehlerquelle.

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[1] Kost 211: Reismehl 666 Teile. Kasein 150 T. Arachisöl 37 T. Dorschlebertran 3 T. Weizenkeimlinge 30 T. Zucker 10 T. Hagebuttenmehl 5 T. Trockenhefe 30 T. Salzgemisch 61,6 T. $NaHCO_3$ 7 T. Insgesamt 1000 T. Salzgemisch: NaCl 83 g, $MgSo_4,7H_2O$ 113 g, KH_2PO_4 100 g, Kalziumlaktat 540 g, $Ca(H_2PO_4)2,H_2O$ 380 g, Ferrizitrat 35 g, MgO 64 g, Totalgewicht: 1315 g.

3. *Gurke enthält eine Askorbinase, die keine anderen reduzierenden Harnbestandteile als Askorbinsäure zerstört.*

Bei Kontrollversuchen mittels des BRIGGS*schen Verfahrens zur Bestimmung von Polyphenolen wird gezeigt, dass auch Urochrom A und Askorbinsäure Phosphormolybdensäure reduzieren.*

Ferner werden die übrigen in dieser Arbeit angewandten quantitativen Bestimmungsverfahren sowie die Methodik der Versuche in vitro und der Tierversuche angegeben.

KAP. III.

Umwandlung zyklischer Verbindungen in vitro und in vivo durch Askorbinsäure unter Bildung von Urochrom A.[1]

I. In vitro.

Die Einwirkung folgender Faktoren auf die Umwandlung von Salizylsäure, Sulfanilamid und Indol ist untersucht worden:

1. Schwankungen des Konzentrationsverhältnisses Askorbinsäure/zyklische Verbindung.
2. Schwankungen des pH.
3. Zusatz verschiedener katalytisch wirksamer Metallionen (zwei- und dreiwertiges Eisen, zweiwertiges Kupfer) in wechselnden Konzentrationen.
4. Die zeitlichen Verhältnisse der Oxydation der Askorbinsäure, der Umwandlung der zyklischen Verbindung und der Farbstoffbildung.
5. Zusatz von mehr als einer zyklischen Verbindung (»Konkurrenzversuche»).
6. Zusatz gewisser zyklischer Hormone, die nach Schrifttumsangaben als Antagonisten des Vitamins C verdächtigt werden können: Prolan, Östron, Cyren, Thyroxin.

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[1] Mit dem Ausdruck »unter Bildung von Urochrom A» ist gemeint, dass Farbstoffe gebildet werden, die sich mittels einer Urochrom-A-Bestimmungsmethode bestimmen und ebenso wie das Urochrom A des Harns isolieren lassen. Der Übersichtlichkeit wegen hat indessen die Untersuchung dieser Urochrom-A-Präparate ein eigenes Kapitel erhalten, in welchem auch das Problem der Einheitlichkeit dieser Stoffe behandelt wird. (Urochrom A: Heilmeyers Terminologie, s. S. 18.)

Schliesslich ist untersucht worden, ob eine Umwandlung einer zyklischen Verbindung stattfindet bei Konzentration der reagierenden Stoffe, die auch in vivo vorkommen können.

Allgemein wurde angestrebt, die Verhältnisse bei der Umwandlung von Indol, Salizylsäure und Sulfanilamid unter möglichst einheitlichen Verhältnissen zu untersuchen. Wegen der Schwerlöslichkeit des Indols war es dabei notwendig, mit relativ schwachen Konzentrationen der reagierenden Stoffe zu arbeiten, und infolgedessen lag die Farbstoffbildung nahe der unteren Grenze der Bestimmungsmöglichkeiten des Urochrom-A-Verfahrens, und in gewissen Fällen waren keine ganz zuverlässigen Werte zu erhalten. Namentlich gilt dies von den Versuchen mit Sulfanilamid, weshalb für diesen Stoff in gewissen Fällen auch andere Konzentrationen gewählt worden sind.

1. Einwirkung von Schwankungen des Konzentrationsverhältnisses Askorbinsäure/zyklische Verbindung auf die Umwandlung der zyklischen Verbindung.

Sowohl mit Natriumsalizylat und Sulfanilamid als mit Indol wurde, wenn eine höhere molare Konzentration der Askorbinsäure als der zyklischen Verbindung gegeben war, eine maximale Umwandlung erzielt (Tab. 32—34). Das optimale Verhältnis ist bei den einzelnen Verbindungen verschieden: für Salizylsäure 16 : 1 (nicht mit grösseren Konzentrationsunterschieden untersucht), für Sulfanilamid 8 : 1, für Indol 4 : 1.

Diese Versuche sind mit konstanter Menge der zyklischen Verbindung und wechselnden Askorbinsäuregaben ausgeführt worden. Behält man statt dessen die Askorbinsäuremenge unverändert bei und verändert die Menge der zyklischen Verbindung, so findet man, wie aus Tabelle 35 hervorgeht, dasselbe: die Umwandlung der zyklischen Verbindung, in Prozent der zugesetzten Menge ausgedrückt, ist bei Askorbinsäure im Überschuss am grössten. Die absolute Menge umgewandelter zyklischer Verbindung dagegen ist bei steigendem Zusatz der zyklischen Verbindung grösser und hält sich, nachdem etwa gleichmolare Konzentration erreicht ist, ungefähr konstant auch

TABELLE 32.

Umwandlung von Natriumsalizylat durch Askorbinsäure. Einwirkung des Zusatzes wechselnder Mengen Askorbinsäure.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 80 mg Natriumsalizylat, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn, pH 5,8.

In Versuch Nr. 6 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrizitrat = 2 : 1 : 0,1.

In Versuch Nr. 9 ist die Umwandlung am umfassendsten und entspricht 67 % der zugesetzten Menge Natriumsalizylat.

Nr.	Zugesetzte Ask. (mg)	Wiedergefunden		Urochrom-A E
		Ask. (mg)	Natr.-sal. (mg)	
1—2	0		79,1	0
3	22		68,5	0,06
4	44		64,0	0,21
5	88		53,5	0,12
6	176	1,5	40,7	0,24
7	352	3,0	33,9	0,34
8	704	8,8	32,4	0,55
9	1408	37,5	26,4	1,32

TABELLE 33.

Umwandlung von Sulfanilamid durch Askorbinsäure. Einwirkung des Zusatzes wechselnder Mengen Askorbinsäure.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 86 mg Sulfanilamid, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. pH 5,8.

In Versuch Nr. 6 ist das molare Konzentrationsverhältnis Askorbinsäure/Sulfanilamid/Ferrizitrat = 2 : 1 : 0,1.

In Versuch Nr. 8 ist die Umwandlung am umfassendsten und entspricht 79,3 % der zugesetzten Menge Sulfanilamid.

Nr.	Zugesetzte Ask. (mg)	Wiedergefunden		Urochrom-A E
		Ask. (mg)	Sulf. (mg)	
1—2	0		82,3	0
3	22	0	71,6	0
4	44	1,0	60,2	0,01
5	88	1,0	51,0	
6	176	1,0	35,6	0,09
7	352	2,0	20,9	0,35
8	704	50,4	17,8	0,69
9	1408	740,0	41,8	0,82

TABELLE 34.

Umwandlung von Indol durch Askorbinsäure. Einwirkung des Zusatzes wechselnder Mengen Askorbinsäure.

Kolbenversuche. Zusatz in sämtlichen Versuchen 29,3 mg Indol, 13,6 mg Ferrizitrat. pH 5,8. Versuchszeit 15,5 Stdn.

In Versuch Nr. 6 ist das molare Konzentrationsverhältnis Askorbinsäure/Indol/Ferrizitrat = 2 : 0,5 : 0,1.

In Versuch Nr. 6 ist die Umwandlung am umfassendsten und entspricht 95,6 % der zugesetzten Indolmenge.

Nr.	Zugesetzte Ask. (mg)	Wiedergefunden		Urochrom-A E
		Ask. (mg)	Indol (mg)	
1—2	0	0	12,6	0
3	22	0	8,2	0,035
4	44	0	4,7	0,04
5	88	0	3,2	0,12
6	176	0	1,3	0,13
7	352	19,0	1,7	0,56
8	704	29,8	2,0	0,97
9	1408	226,0	5,4	1,13

bei Zusatz eines sehr hohen Überschusses der zyklischen Verbindung. (Der Versuch hat aus Gründen der Löslichkeit nur mit Natriumsalizylat ausgeführt werden können.)

In den Fällen, in denen die Askorbinsäure nicht vollständig oxydiert worden ist (Sulfanilamid und Indol, Tab. 32 und 34), wird die Umwandlung bei Anwesenheit nicht-oxydierter Askorbinsäure gehemmt. Eine Reihe von Schalenversuchen lässt dies noch deutlicher erkennen (Abb. 7). Von Interesse ist in diesem Zusammenhang ferner, dass die Oxydation der Askorbinsäure, was ihre Schnelligkeit angeht, gleich schnell verläuft, unabhängig davon, ob eine zyklische Verbindung (Sulfanilamid) anwesend ist oder nicht.

In den Kolbenversuchen, in denen die Urochrom-A-Bestimmungen in der gewohnten Weise durchgeführt wurden, entsprechen die Urochrom-A-Werte in der Hauptsache der Umwandlung der zyklischen Verbindung, ohne dass jedoch eine

TABELLE 35.

Umwandlung von Natriumsalizylat durch Askorbinsäure. Einwirkung des Zusatzes wechselnder Natriumsalizylatmengen zu einer gleichbleibenden Menge Askorbinsäure.

Kolbenversuche. Zusatz in sämtlichen Versuchen 176 mg Askorbinsäure, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. pH 5,8.

In Versuch Nr. 4 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrizitrat = 2 : 1 : 0,1.

In Versuch Nr. 1 ist die prozentuale Umwandlung am umfassendsten und entspricht 88,7 % der zugesetzten Menge Natriumsalizylat.

In Versuch Nr. 10 ist die höchste absolute Umwandlung zu verzeichnen, 10,2 % der zugesetzten Natriumsalizylatmenge entsprechend.

In sämtlichen Versuchen wird die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes Natr.-sal. (mg)	Umgewandeltes Natr.-sal. (mg)	Urochrom-A E
1	10	8,87	0,02
2	20	15,25	0,13
3	40	24,9	0,10
4	80	37,8	0,10
5	120	38,6	0,22
6	160	44,7	0,25
7	240	50,0	0,32
8	320	48,6	0,45
9	480	54,7	0,55
10	640	65,4	0,50
11	1280	40,4	0,42

direkte Proportionalität zwischen der umgewandelten zyklischen Verbindung und dem gebildeten Farbstoff bestände. Dies geht u. a. daraus hervor, dass die Hemmung der Umwandlung der zyklischen Verbindung in Anwesenheit von nicht-oxydierter Askorbinsäure keine Hemmung der Urochrom-A-Bildung im Gefolge hat.

In den Schalenversuchen, in denen nur die »Gesamtfarbstoffbildung» bestimmt werden konnte (siehe S. 90), wird diese dagegen bei Askorbinsäure im Überschuss hochgradig gehemmt.

In mehreren Fällen ist die Askorbinsäure auch nach vorheriger Behandlung mit Schwefelwasserstoff bestimmt worden,

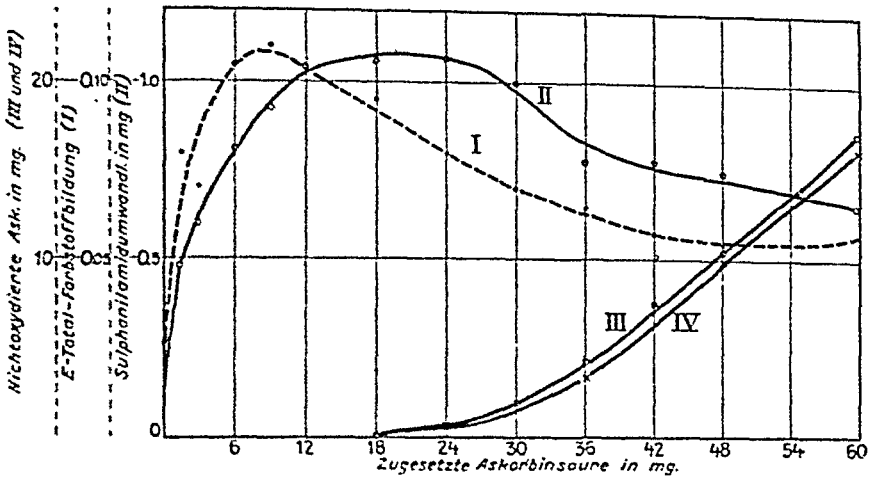

Abb. 7. *Hemmung von Sulfanilamidumwandlung und Total-Farbstoff-Bildung durch Überschuss von Askorbinsäure.*

Schalenversuche. Zugesetzt: 2 mg Sulfanilamid, 1,86 mg Ferrizitrat 10 ccm Phosphatpufferlösung, pH 5,8. (Zu den Versuchen Kurve IV entsprechend kein Sulfanilamidzusatz.) Gesamtvolumen 17,5 ccm, Versuchszeit 21 Stdn.

um also etwa reversibel oxydierte Askorbinsäure zu erfassen. Die Unterschiede zwischen diesen Bestimmungen und den bei direkter Titration mit dem Reagens gefundenen Werten waren jedoch so geringfügig, dass es unnötig erschien, durchgängig sowohl reversibel als irreversibel oxydierte Askorbinsäure zu bestimmen.

Sowohl Sulfanilamid als Salizylsäure wird praktisch quantitativ in den Kontrollversuchen wiedergefunden, während Indol kaum in der Hälfte der zugesetzten Menge wiedergefunden wird. Dies dürfte sich ganz einfach dadurch erklären, dass die Belüftung eine Destillation des Indols bewirkt.

Bei der quantitativen Bestimmung des restlichen Sulfanilamids bzw. der restlichen Salizylsäure nach beendigtem Versuch wurde bei direkter Bestimmung wie nach Hydrolyse dasselbe Resultat erzielt. Die Umwandlung kann also nicht in einer Paarung der zyklischen Verbindung mit der Askorbinsäure bestanden haben.

Die Sauerstoffzufuhr (durchströmende Luft) war notwendig, damit eine Umwandlung erfolgte.

Das Urochrom A konnte in Umwandlungsversuchen mit sämtlichen untersuchten zyklischen Verbindungen aus der betreffenden Versuchslösung isoliert werden.

2. Die Einwirkung von Schwankungen des pH auf die Umwandlung zyklischer Verbindungen durch Askorbinsäure.

Es erwies sich als unmöglich, die Untersuchung auf den gesamten pH-Bereich auszudehnen, da beim Übergang beispielsweise von Phosphatpuffer zu Pufferlösung mit Borat Schwankungen des Umwandlungsergebnisses auftraten, die sich nicht durch die Veränderungen des pH erklären liessen. Die Untersuchungen wurden daher auf den pH-Bereich beschränkt, in welchem es möglich ist, mit Phosphatpuffer zu arbeiten.

Wie aus Tabelle 36 hervorgeht, bedingen die pH-Schwankungen nur verhältnismässig kleine Variationen des Umwandlungsergebnisses, während die Bildung des Urochroms A stärker beeinflusst wird. Bei allen untersuchten zyklischen Verbindungen konnte festgestellt werden, dass ein Optimum in bezug auf die Umwandlung bei pH $5{,}_4$—$5{,}_8$ und in bezug auf die Urochrom-A-Bildung bei etwa $6{,}_6$ liegt.

Für die übrigen Versuche in vitro ist im allgemeinen pH $5{,}_8$ gewählt worden.

3. Die Einwirkung dreiwertigen und zweiwertigen Eisens sowie zweiwertigen Kupfers auf die Umwandlung zyklischer Verbindungen durch Askorbinsäure.

In ihren Untersuchungen über die oxydative Desaminierung des Histidins konnten Edlbacher und v. Segesser (1937 a) zeigen, dass dieser Vorgang durch dreiwertiges Eisen (Ferrisulfat), Hämin oder Hämoglobin katalysiert wurde. Ohne Katalysator fand praktisch keine Desaminierung statt.

Hier ist die Einwirkung einiger Metalle auf die Umwandlung von Natriumsalizylat, Sulfanilamid und Indol untersucht worden. Wie Edlbacher und v. Segesser habe ich die Einwirkung dreiwertigen Eisens untersucht, doch habe ich statt des Ferri-

TABELLE 36.

Umwandlung von Natriumsalizylat, Sulfanilamid und Indol durch Askorbinsäure. Einwirkung wechselnder pH-Werte.

Kolbenversuche. Molares Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrizitrat = 2 : 1 : 0,1; Askorbinsäure/Sulfanilamid/Ferrizitrat = 4 : 2 : 0,1; Askorbinsäure/Indol/Ferrizitrat = 2 : 0,5 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	pH	*Natriumsalizylat*		*Sulfanilamid*		*Indol*	
		Wied. gef. Natr. sal. (mg)	Urochr. A E	Wied. gef. Sulf. (mg)	Urochr. A E	Wied. gef. Indol (mg)	Urochr. A E
1	5,4	43,0	0,13	93,0	0,43	0,8	0,095
2	5,8	43,7	0,15	93,0	0,42	2,7	0,20
3	6,2	41,5	0,17	100,8	0,45	1,9	0,28
4	6,6	43,0	0,16	100,8	0,48	1,7	0,32
5	7,0	47,5	0,10	105,4	0,49	4,7	0,075
6	7,4	52,8	0,06	111,6	0,26	5,3	0,075
7	7,8	55,0	0,15	124,0	0,35	11,2	0,15
8	8,2	55,8		120,9	0,23	5,2	0,07
Zusatz von		Natriumsalizylat		Sulfanilamid		Indol	
		80 mg		172 mg		29,3 mg	
Zugesetzte Askorbinsäure		176 mg		352 mg		176 mg	
Zugesetztes Ferrizitrat		13,6 mg		13,6 mg		13,6 mg	
Versuchszeit		17 Stdn.		20 Stdn.		18,5 Stdn.	
Maximale Umwandlung in %		48,1		45,9		97,4	

sulfats, das mit Phosphatpuffer eine Fällung ergibt, Ferrizitrat verwendet. Vergleichshalber ist die Einwirkung zweiwertigen Eisens (Ferrochlorids) sowie ausserdem, in Anbetracht der stark katalysierenden Wirkung von Kupfer auf die Oxydation von Askorbinsäure, die von Kupferazetat untersucht worden.

Die Versuchsergebnisse sind in Tabelle 37—39 wiedergegeben, eine Zusammenfassung der Ergebnisse bietet Tabelle 40.

TABELLE 37.

Umwandlung von Natriumsalizylat, Sulfanilamid und Indol durch Askorbinsäure. Einwirkung wechselnder Zusätze von Ferrizitrat.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure. pH 5,8.

In Versuch Nr. 6 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrizitrat und Askorbinsäure/Sulfanilamid/Ferrizitrat = 2 : 1 : 0,1, das Verhältnis Askorbinsäure/Indol/Ferrizitrat = 2 : 0,5 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetzt. Ferrizitrat (mg)	*Natriumsalizylat*		*Sulfanilamid*		*Indol*	
		Wied. gef. Natr. sal. (mg)	Urochr. A E	Wied. gef. Sulf. (mg)	Urochr. A E	Wied. gef. Indol (mg)	Urochr. A E
1–2	0	59,5	0,12	62,1	0,0	10,9	0,085
3	0,027	61,7	0,09	61,2	0,02	8,2	0,05
4	0,27	62,9	0,12	56,1	0,01	4,8	0,08
5	2,72	49,7	0,20	46,6	0,04	2,7	0,20
6	13,6	43,7	0,21	43,0	0,02	1,0	0,11
7	27,2	41,4	0,16	39,0	0,07	0,6	0,17
8	54,4	39,9	0,17	39,0	0,03	1,5	0,18
Zusatz von		Natriumsalizylat 80 mg		Sulfanilamid 86 mg		Indol 29,3 mg	
Versuchszeit		17 Stdn.		17 Stdn.		15,5 Stdn.	

Es zeigt sich, dass sämtliche untersuchten Metallsalze die Umwandlung zyklischer Verbindungen durch Askorbinsäure steigern. Die Versuche wurden bei einem pH von 5,8 ausgeführt, ähnliche Ergebnisse wurden aber auch bei einem pH von 7 gefunden. Es zeigt sich jedoch auch, dass im Gegensatz zu den von EDLBACHER und v. SEGESSER gemachten Feststellungen eine Umwandlung der zyklischen Verbindungen auch ohne Zusatz eines katalytisch wirksamen Metalls stattfinden kann. In einem früheren Abschnitt ist gezeigt worden, dass man unter den gegebenen Bedingungen die zugesetzten Mengen Natriumsalizylat und Sulfanilamid quantitativ wiederfindet, wenn keine Askorbinsäure im Umwandlungsversuch zugegen ist, weshalb es sich hier also um eine Askorbinsäurewirkung handeln muss.

TABELLE 38.

Umwandlung von Natriumsalizylat, Sulfanilamid und Indol durch Askorbinsäure. Einwirkung wechselnder Zusätze von Ferrochlorid.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure. pH 5,8.

In Versuch Nr. 6 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrochlorid und Askorbinsäure/Sulfanilamid/Ferrochlorid = 2 : 1 : 0,1, das Verhältnis Askorbinsäure/Indol/Ferrochlorid = 2 : 0,5 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes Ferrochlorid (mg)	*Natriumsalizylat*		*Sulfanilamid*		*Indol*	
		Wied. gef. Natr. sal. (mg)	Urochr. A E	Wied. gef. Sulf. (mg)	Urochr. A E	Wied. gef. Indol (mg)	Urochr. A E
1–2	0	61,4	0,1	55,6	0,04	13,6	0,085
3	0,0127	58,7	0,14	57,1	0,06	9,8	0,08
4	0,127	61,0	0,04	52,8	0,02	9,7	0,175
5	1,27	55,0	0,13	46,7	0,01	4,7	0,17
6	6,35	54,2	0,26	49,1		6,8	0,10
7	12,7	58,7	0,12	52,2	0,01	6,6	0,05
8	25,4	62,5	0,06	49,1	0,10	2,3	0,155
Zusatz von		Natriumsalizylat 80 mg		Sulfanilamid 86 mg		Indol 29,3 mg	
Versuchszeit		17 Stdn.		17 Stdn.		16 Stdn.	

Bei Zusatz eines Katalysators machte sich ein deutlicher Unterschied zwischen dem mit den Benzolverbindungen, Natriumsalizylat und Sulfanilamid, erzielten Ergebnis einerseits und dem mit Indol gewonnenen andererseits bemerkbar. Bei den Benzolverbindungen wurde mit Ferrizitrat eine weit stärkere Wirkung als mit den übrigen erzielt, während bei Indol alle geprüften Verbindungen dieselbe Wirkung hatten.

Bei Indol ist ausserdem die niedrigste molare Katalysatorkonzentration, bei der eine optimale Wirkung beobachtet wird,

TABELLE 39.

Umwandlung von Natriumsalizylat, Sulfanilamid und Indol durch Askorbinsäure. Einwirkung wechselnder Zusätze von Kupferazetat.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure. pH 5,8.

In Versuch Nr. 6 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Kupferazetat und Askorbinsäure/Sulfanilamid/Kupferazetat = 2 : 1 : 0,1, das Verhältnis Askorbinsäure/Indol/Kupferazetat = 2 : 0,5 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetzt. Kupf. azet. (mg)	*Natriumsalizylat*		*Sulfanilamid*		*Indol*	
		Wied. gef. Natr. sal. (mg)	Urochr. A E	Wied. gef. Sulf. (mg)	Urochr. A E	Wied. gef. Indol (mg)	Urochr. A E
1–2	0	61,4	0,10	62,1	0,01	13,6	0,085
3	0,018	64,7	0,07	61,8	0,0	6,4	0,065
4	0,18	64,7	0,06	63,0	0,03	8,2	0,08
5	1,82	58,0	0,16	59,3	0,01	5,2	0,25
6	9,08	58,0	0,19	57,5	0,04	2,0	0,24
7	18,16	58,0	0,27	55,1	0,08	3,0	0,22
8	36,32	58,0	0,28	55,1	0,04	1,4	0,24
Zusatz von		Natriumsalizylat 80 mg		Sulfanilamid 86 mg		Indol 29,3 mg	
Versuchszeit		17 Stdn.		17 Stdn.		16 Stdn.	

für alle Katalysatoren etwa dieselbe. Bei Natriumsalizylat dagegen sinkt diese Konzentration wesentlich vom Ferrizitrat über Ferrochlorid bis zu einem sehr niedrigen Gehalt des Kupferazetats.

Die Werte der Urochrom-A-Bildung sind in der Übersichtstabelle nicht angegeben, doch zeigen die ursprünglichen Tabellen (37—39), dass im grossen ganzen eine maximale Farbstoffbildung bei derselben Katalysatorkonzentration, die eine maximale Umwandlung ergab, gefunden wurde.

TABELLE 40.

Zusammenfassung der Ergebnisse von Tabelle 37—39.

In dieser Tabelle ist für jeden geprüften Katalysator und jede geprüfte zyklische Verbindung angegeben:

unter »K» die Umwandlung der zyklischen Verbindung in den Kontrollversuchen (also mit Askorbinsäure allein), ausgedrückt in Prozent der zugesetzten Menge,

unter »+ Fe», »+ Fe», »+ Ku» die maximale Umwandlung der zyklischen Verbindung, ausgedrückt in Prozent der zugesetzten Menge, bei Zusatz eines Katalysators,

unter »Ask/Fe», »Ask/Fe», »Ask/Ku» das molare Konzentrationsverhältnis Askorbinsäure/Katalysator in dem Versuch, in welchem maximale Umwandlung der zyklischen Verbindung gefunden wurde.

	Ferrizitrat			Ferrochlorid			Kupferazetat		
	K	+ Fe	Ask/Fe	K	+ Fe	Ask/Fe	K	+ Ku	Ask/Ku
Natriumsalizylat	25,6	51,1	2/0,4	23,2	32,2	2/0,1	23,2	27,5	2/0,02
Sulfanilamid	27,8	54,7	2/0,2	35,3	45,7	2/0,02	27,8	35,9	2/0,2
Indol	62,8	97,9	2/0,2	53,6	92,2	2/0,4	53,7	95,2	2/0,4

4. Die Oxydation von Askorbinsäure, die Umwandlung der aromatischen Verbindung und die Farbstoffbildung im Verhältnis zum Faktor der Zeit.

Für diese Versuche ist das Indol weniger geeignet, da bei der langen Dauer der Versuche die Flüchtigkeit des Indols allzu grossen Einfluss auf das Ergebnis haben dürfte.

Wie aus Abb. 8 (Kolbenversuch mit Natriumsalizylat) hervorgeht, findet zuerst eine reversible Oxydation der Askorbinsäure statt, auf die jedoch fast unmittelbar eine irreversible Oxydation folgt. Daran schliesst sich eine Umwandlung der zyklischen Verbindung an, die, wenn alle Askorbinsäure irreversibel oxydiert ist, etwa nur zur Hälfte vollendet ist. Zuletzt tritt eine Farbstoffbildung ein, die, wenn alle Askorbinsäure irreversibel oxydiert ist, erst etwa ein Drittel ihres Endwertes erreicht hat und die noch andauert, nachdem die Umwandlung der zyklischen Verbindung ihr Maximum erreicht hat.

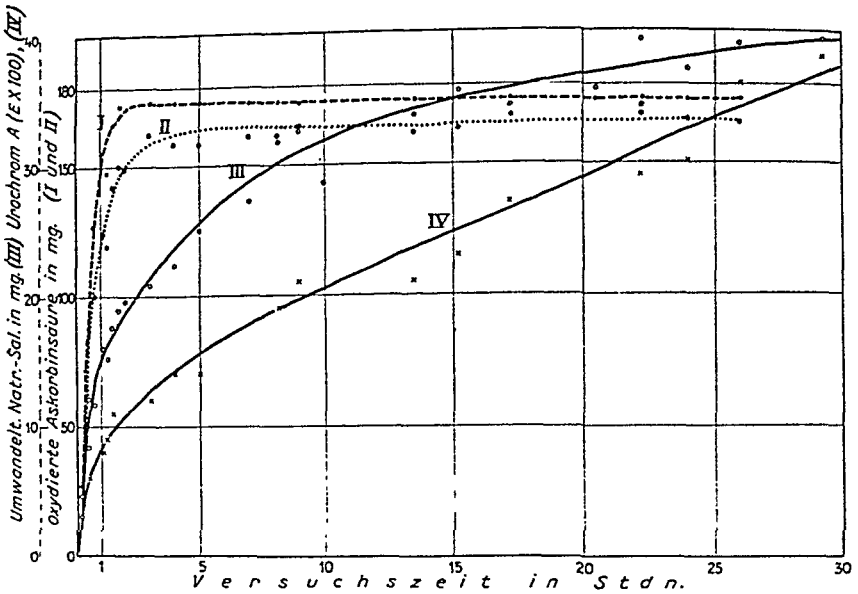

Abb. 8. *Das Verhältnis zwischen der Oxydation von Askorbinsäure, der Umwandlung von Natriumsalizylat, der Urochrom-A-Bildung und der Versuchszeit.*

Kolbenversuche. Zugesetzt: 176 mg Askorbinsäure, 80 mg Natriumsalizylat, 13,6 mg Ferrizitrat. pH 5,8.

Kurve I Askorbinsäurebestimmung ohne, Kurve II mit H_2S-Behandlung.

Das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrizitrat = 2 : 1 : 0,1.

Ähnlich war das Ergebnis bei einem Schalenversuch mit Sulfanilamid (Abb. 9). Wahrscheinlich weil die Sauerstoffzufuhr bei diesen Versuchen weniger effektiv ist, vollzieht sich die Oxydation der Askorbinsäure langsamer, und sämtliche Teilreaktionen verlaufen paralleler.

In beiden Versuchen über die zeitliche Folge ist die Askorbinsäure nach Tillmans (siehe S. 78) mit und ohne Vorbehandlung mit Schwefelwasserstoff bestimmt worden. Die nach Vorbehandlung mit Schwefelwasserstoff gemachten Bestimmungen zeigen, dass auch nach beendigtem Versuch noch eine gewisse Menge der reduzierenden Verbindung übrig ist. (In Abb. 8 und 9 treffen die beiden Askorbinsäurekurven einander nie.) Aus ähnlichen Versuchen mit Bestimmung nach Tauber (siehe S. 78) geht indes-

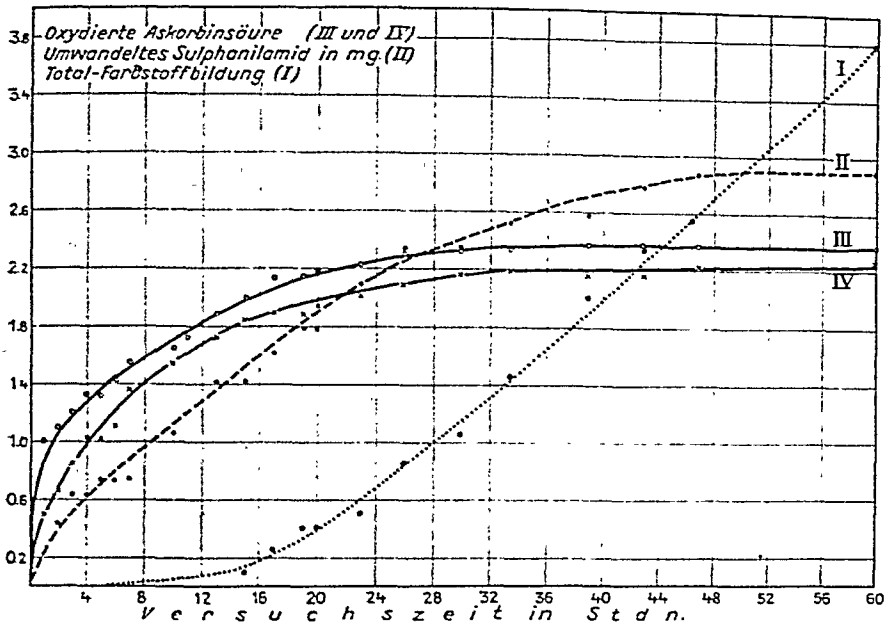

Abb. 9. *Das Verhältnis zwischen der Oxydation der Askorbinsäure, der Umwandlung von Sulfanilamid, der Total-Farbstoffbildung und der Versuchszeit.*

Schalenversuche. Zugesetzt: 4 mg Sulfanilamid, 24 mg Askorbinsäure, 1,36 mg Ferrizitrat, 10 ccm Phosphatpuffer. pH 5,8. Gesamtvolumen 20 ccm.

Kurve III Askorbinsäurebestimmung ohne, Kurve IV mit H_2S-Behandlung.

Die Zahlenwerte der Ordinate mit 10 multipliziert geben »Oxydierte Askorbinsäure» in mg, mit 10 dividiert die Extinktionswerte der »Totalfarbstoffbildung» an.

sen hervor, dass es sich bei der fraglichen Verbindung nicht um restliche, nicht oxydierte Askorbinsäure, sondern wahrscheinlich um Urochrom A handelt.

5. Hemmung der Umwandlung einer zyklischen Verbindung durch Askorbinsäure bei Zufuhr einer anderen zyklischen Verbindung (»Konkurrenzversuche»).

Die Voraussetzung dafür, dass ein Konkurrenzversuch mit zwei zyklischen Verbindungen angestellt werden kann, dürfte sein, dass beide während des Versuchs nur durch einen Faktor beeinflusst werden, nämlich die Umwandlung durch Askorbin-

säure. Man muss sie also in den Kontrollversuchen ohne Zusatz von Askorbinsäure quantitativ wiederfinden.

Diese Voraussetzung ist bei Natriumsalizylat und Sulfanilamid gegeben. Ein Konkurrenzversuch mit diesen beiden Verbindungen wurde folgendermassen durchgeführt: teils wurde die Einwirkung der Zufuhr von Natriumsalizylat auf eine Versuchsreihe mit konstantem Sulfanilamidzusatz untersucht, teils umgekehrt die Einwirkung von Sulfanilamid auf eine Versuchsreihe mit konstantem Natriumsalizylatzusatz. In sämtlichen Fällen wurden Sulfanilamid- wie Salizylsäurebestimmungen gemacht.

Wie aus Tabelle 41 hervorgeht, zeigt es sich, dass in erster Linie das Sulfanilamid umgewandelt wird, danach die Salizylsäure. Dies äussert sich auf zweierlei Weise: erstens ist die Hemmung der Sulfanilamidumwandlung bei Zusatz von Salizylsäure geringer als die Hemmung der Salizylsäureumwandlung durch Sulfanilamid. (Siehe die Spalte »Umgewandelte Mengen in % des Ergebnisses der Kontrolle».) Zweitens findet man eine stärkere Umwandlung der zur Hemmung der Salizylsäureumwandlung zugesetzten Sulfanilamidmengen als der Salizylsäuremengen, die zur Hemmung der Sulfanilamidumwandlung eingesetzt wurden. (Siehe die Spalte Umgewandelte Mengen in % der zugesetzten Menge».)

Die Erklärung dafür, dass die Umwandlung einer zyklischen Verbindung durch die Anwesenheit einer zweiten gehemmt werden kann, ist selbstverständlich die, dass beide durch Askorbinsäure umgewandelt werden können und dass bei einem bestimmten Askorbinsäurezusatz nur eine bestimmte Gesamtwandlung zyklischer Verbindungen stattfinden kann. In welchem Verhältnis die beiden Verbindungen an der Gesamtwandlung beteiligt sind, muss an einer wechselnden Affinität zum Umwandlungsprozess liegen.

In Übereinstimmung mit dem in Tabelle 35 (S. 98) wiedergegebenen Ergebnis, wonach in einer Versuchsreihe mit gleichbleibendem Askorbinsäurezusatz, doch steigendem Zusatz von Natriumsalizylat in gewissen Grenzen eine Steigerung der Gesamtmenge der umgewandelten zyklischen Verbindung eintrat, wurde ein ähnliches Resultat hier erhalten. In beiden Fällen

TABELLE 41.

Umwandlung von Natriumsalizylat und Sulfanilamid durch Askorbinsäure. (»Konkurrenzversuch».)

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. pH 5,8.

In den Versuchen 4 und 9 ist das molare Konzentrationsverhältnis Askorbinsäure/Sulfanilamid/Natriumsalizylat/Ferrizitrat = 2 : 1 : 1 : 0,1

(N.-s. = Natriumsalizylat; Sulf. = Sulfanilamid; K = Kontrollversuch.)

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes		Umgewandelt.		Umgewandelte Mengen in % des Ergebn. der Kontrolle		Umgewandelte Mengen in % der zugesetzten Menge		Urochrom-A E
	N.-s. (mg)	Sulf. (mg)	N.-s. (mg)	Sulf. (mg)	N.-s.	Sulf.	N.-s.	Sulf.	
1–2 (K)		86		39,3		(100)		45,7	0,00
3	40	86	11,0	27,4		69,7	27,5		0,18
4	80	86	20,7	26,7		67,9	25,9		0,27
5	160	86	39,7	23,5		59,8	24,8		0,49
6–7 (K)	80		36,1		(100)		45,1		0,18
8	80	43	27,2	19,8	75,3			46,0	0,27
9	80	86	17,0	29,3	47,1			34,1	0,37
10	80	172	9,9	49,6	27,4			28,8	0,33

ist ferner eine vermehrte Urochrom-A-Bildung mit steigendem Zusatz der zyklischen Verbindung zu verzeichnen.

Weitere Beispiele einer Hemmung der Umwandlung bieten die Tabellen 42—43: die Umwandlung von Indol kann durch Zusatz von Salizylsäure, die Umwandlung von Salizylsäure durch Zusatz von Indol gehemmt werden.

Um einen Vergleich mit gewissen Versuchen in vivo (S. 135) zu ermöglichen, ist ausserdem ein Versuch angegeben, in welchem gezeigt wird, dass die Umwandlung von Histidin durch Zusatz von Phenol gehemmt werden kann (Tab. 44).

TABELLE 42.

Umwandlung von Natriumsalizylat durch Askorbinsäure. Einwirkung bei Zusatz wechselnder Indolmengen.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 80 mg Natriumsalizylat, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. *pH* 5,8.

In Versuch Nr. 5 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Indol/Ferrizitrat = 2 : 1 : 1 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes Indol (mg)	Wiedergefundenes Natr.-sal. (mg)	Urochrom-A E
1—2	0	42,2	0,23
3	14,65	45,2	0,19
4	29,3	58,8	0,21
5	58,6	61,1	0,26
6	117,2	64,8	0,37

TABELLE 43.

Umwandlung von Indol durch Askorbinsäure. Einwirkung durch Zusatz von Natriumsalizylat.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 13,6 mg Ferrizitrat. Versuchszeit 18,5 Stdn. pH 5,8.

In Versuch Nr. 4 ist das molare Konzentrationsverhältnis Askorbinsäure/Natriumsalizylat/Ferrizitrat = 2 : 0,5 : 0,5 : 0,1.

Die Askorbinsäure wurde in sämtlichen Versuchen vollständig oxydiert.

Nr.	Zugesetzt (mg)		Wiedergefunden (mg)		Urochrom-A E
	Indol	Natr.-sal.	Indol	Natr.-sal.	
1	29,3	10	2,0	4,3	0,08
2	29,3	20	2,9	9,2	0,34
3	29,3	30	4,1	15,9	0,27
4	29,3	40	4,8	20,7	0,24
5	29,3	80	6,1	47,2	0,42
6	29,3		1,9		0,15
7	29,3		1,8		0,12
8		40		23,1	0,22
9		40		23,1	0,22

TABELLE 44.

Umwandlung von Histidin durch Askorbinsäure. Einwirkung durch Zusatz von Phenol.

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 209,5 mg Histidin, 27,2 mg Ferrizitrat. Versuchszeit 20 Stdn. pH 5,8. In Versuch Nr. 4 ist das molare Konzentrationsverhältnis Askorbinsäure/Histidin/Pheonol/Ferrizitrat = 1 : 1 : 1 : 0,2

Nr.	Zugesetztes Phenol (mg)	Wiedergefundenes Histidin (mg)	Urochrom-A E
1—2		33,0	0,93
3	18,8	44,9	0,96
4	94,0	68,6	0,95
5	188,0	73,9	0,80

6. Die Umwandlung einer zyklischen Verbindung durch Askorbinsäure lässt sich durch gewisse Hormone mit zyklischer Konstitution beeinflussen.

Im vorigen Abschnitt wurde gezeigt, dass die Umwandlung einer zyklischen Verbindung durch Askorbinsäure von einer anderen zyklischen Verbindung beeinflusst (gehemmt) werden kann, was daran liegt, dass beide umgewandelt werden. Die Gesamtmenge umgewandelter zyklischer Verbindung dürfte also am ehesten etwas grösser sein, wie z. B. die gesteigerte Farbstoffbildung erkennen lässt.

Von mehreren zyklischen Verbindungen mit hohem physiologischem Interesse, vor allem gewissen Hormonen, nimmt man an, sie stünden in einem Gegensatzverhältnis zum Vitamin C (s. Kap. I). Es erschien daher wünschenswert, die Einwirkung solcher Verbindungen auf die Umwandlung einer anderen zyklischen Verbindung durch Askorbinsäure zu untersuchen. Es liesse sich denken, dass eine etwaige Hemmung durch ein solches Hormon analog den vorerwähnten Ergebnissen andeuten würde, dass eine Umwandlung auch des Hormons stattgefunden hätte. Im gleichen Sinne würde selbstverständlich eine Steigerung der Farbstoffbildung sprechen.

TABELLE 45.

Umwandlung von Sulfanilamid durch Askorbinsäure. Einwirkung durch Zusatz von Östron und Cyren B (Bayer).

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 43 mg Sulfanilamid, 6,8 mg Ferrizitrat und 20 ccm 96 % Alkohol. Versuchszeit 17 Stdn. pH 5,8.

In sämtlichen Versuchen ist das molare Konzentrationsverhältnis Askorbinsäure/Sulfanilamid/Ferrizitrat = 2 : 0,5 : 0,05.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert. Die Hormonpräparate wurden in Alkohol gelöst.

Nr.	Zugesetztes Hormon (mg)	Oxydiertes Sulf.-am. (mg)	Oxydiertes Sulf.-am. in % der Kontrolle
1–2 (K)		14,3	(100)
	Östron		
3	2	3,7	25,9
4	10	8,9	62,2
	Cyren		
5	2	3,7	25,9
6	10	5,0	35,0

TABELLE 46.

Umwandlung von Natriumsalizylat durch Askorbinsäure. Einwirkung durch Zusatz wechselnder Mengen Prolan (Bayer).

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 80 mg Natriumsalizylat, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. pH 5,8.

Das molare Konzentrationsverhältnis: Askorbinsäure/Natriumsalizylat/Ferrizitrat = 2 : 1 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes Prolan (mg)	Wiedergefundenes Natr.-sal. (mg)	Urochrom-A E
1—2	0	41,1	0,48
3	20	41,5	0,55
4	40	36,9	0,49
5	80	42,2	0,56
6	115	30,9	0,40

TABELLE 47.

Umwandlung von Sulfanilamid durch Askorbinsäure. Einwirkung des Zusatzes wechselnder Mengen Prolan (Bayer).

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 86 mg Sulfanilamid, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. pH 5,8.

In sämtlichen Versuchen ist das molare Konzentrationsverhältnis Askorbinsäure/Sulfanilamid/Ferrizitrat = 2 : 1 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes Prolan (mg)	Wiedergefundenes Sulf. (mg)	Urochrom-A E
1	0	52,2	0
2	10	49,0	0
3	20	47,7	0,05
4	40	47,0	0
5	80	41,2	0
6	160	39,9	0

TABELLE 48.

Umwandlung von Indol durch Askorbinsäure. Einwirkung durch Zusatz wechselnder Mengen Prolan (Bayer).

Kolbenversuche. Zusatz in sämtlichen Versuchen: 176 mg Askorbinsäure, 29,3 mg Indol, 13,6 mg Ferrizitrat. Versuchszeit 17 Stdn. pH 5,8.

Das molare Konzentrationsverhältnis Askorbinsäure/Indol/Ferrizitrat ist in sämtlichen Versuchen 2 : 0,5 : 0,1.

In sämtlichen Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zugesetztes Prolan (mg)	Wiedergefundenes Indol (mg)	Urochrom-A E
1—2	0	1,4	0,045
3	5	1,0	0,05
4	20	0,8	0,09
5	40	2,5	0,10
6	80	1,0	0,14

TABELLE 49.

Umwandlung von Sulfanilamid durch Askorbinsäure. Einwirkung durch Zusatz von Thyroxin.

Schalenversuche. Zusatz in sämtlichen Versuchen: 7,04 mg Askorbinsäure, 1,72 mg Sulfanilamid, 0,136 mg Ferrizitrat. Versuchszeit 7 Stdn. pH 5,8. Gesamtvolumen 16 ccm.

In allen Versuchen ist das molare Konzentrationsverhältnis Askorbinsäure/Sulfanilamid/Ferrizitrat = 8 : 2 : 0,1.

Nr.	Zugesetztes Thyroxin in mg	Umgewandeltes Sulfanilamid in mg	Umgewandeltes Sulfanilamid in % der Kontrolle
1—2 (K)		0,57	(100)
3	0,5	0,39	68,4
4	0,1	0,60	105,3

Untersucht wurden hier Prolan, Östron, Cyren B und Thyroxin (Ergebnisse in Tab. 45—49).

Da die Beschaffung genügender Hormonmengen Schwierigkeiten bereitete, sind diese Versuche im allgemeinen mit so kleinen Mengen der reagierenden Stoffe ausgeführt worden, dass Urochrom-A-Bestimmungen unmöglich waren.

Vom Prolan abgesehen, ist in sämtlichen Fällen eine Hemmung des Sulfanilamidabbaus beobachtet worden, und diese Hemmung trat oft auch bei im Vergleich zum Sulfanilamidzusatz niedrigen Hormonkonzentrationen in Erscheinung. Dies gilt vor allem für Östron und Cyren. Prolan übt im allgemeinen keine Einwirkung aus, doch konnte in den höchsten Konzentrationen statt dessen eine Steigerung der Salizylsäure- und Sulfanilamidumwandlung beobachtet werden.

7. Die Umwandlung einer zyklischen Verbindung durch Askorbinsäure kann auch bei sehr niedrigen Konzentrationen der reagierenden Stoffe stattfinden.

In den geschilderten Versuchen in vitro habe ich im allgemeinen mit so hohen Konzentrationen der reagierenden Stoffe gearbeitet, wie sie im lebenden Organismus nicht vorkommen können. Die Konzentrationen der zyklischen Verbindung waren

TABELLE 50.

Umwandlung einer aromatischen Verbindung durch Askorbinsäure kann auch bei sehr schwacher Konzentration der reagierenden Verbindungen eintreten.

Schalenversuche. Versuchszeit 24 Stdn. pH 5,8. Katalysator: Ferrizitrat.

Für jeden Versuch ist ein Kontrollversuch mit Sulfanilamid und Ferrizitrat allein gemacht worden.

Das molare Konzentrationsverhältnis(Askorbinsäure)/Sulfanilamid/Ferrizitrat ist in allen Versuchen (8 :)2 : 0,1.

Nr.	1	2	3	4	5
Sulfanilamid in mg %	11,4	5,7	2,28	1,14	0,57
Askorbinsäure in mg %	47,0	23,5	9,4	4,7	2,35
Umwandlung von Sulfanilamid in % des Ergebnisses der Kontrolle	48,8	49,0	50,0	56,7	47,4

meistens etwa 100—200 mg%, die der Askorbinsäure etwa 300—500 mg%.

Wie aus einem Schalenversuch (Tab. 50) mit Sulfanilamid hervorgeht, kann man indessen noch bei einer Askorbinsäurekonzentration von 2,35 mg% und einer Sulfanilamidkonzentration von 0,57 mg% dieselbe prozentuale Umwandlung des Sulfanilamids beobachten wie bei den höheren Konzentrationen mit gleicher Versuchsanlage. Die Ergebnisse stimmen ausserdem gut mit den in den Kolbenversuchen erzielten überein.

Allen Anzeichen nach würden ähnliche Resultate auch bei noch schwächeren Konzentrationen erhalten werden. Das angewandte Verfahren ist jedoch für Bestimmungen in so stark verdünnten Versuchslösungen ungeeignet.

Die schwächste hier untersuchte Konzentration ist dieselbe, die unter physiologischen Bedingungen vorkommen kann, was für die Möglichkeiten, die In-vitro-Versuche in vivo zu wiederholen, von wesentlicher Bedeutung ist.

Zusammenfassung der Versuchsergebnisse.

Im wesentlichen dürften die Versuche in vitro Folgendes erbracht haben:

1. In Anwesenheit von Askorbinsäure im molaren Überschuss erfolgt eine maximale Umwandlung einer bestimmten Menge zyklischer Verbindung. Für maximale Ausnützung einer bestimmten Askorbinsäuremenge bedarf es einer wenigstens gleichmolaren Konzentration der zyklischen Verbindung.

Ist die Askorbinsäurekonzentration so gross, dass die Askorbinsäure im Laufe des Versuchs nicht oxydiert wird, so tritt eine Hemmung der Umwandlung und der totalen Farbstoffbildung ein. Die Bildung von Urochrom A dagegen wird nicht gehemmt.

Die Oxydation der Askorbinsäure verläuft unverändert schnell, mag nun eine zyklische Verbindung (Sulfanilamid) anwesend sein oder nicht.

Die Umwandlung ist keine Koppelungsreaktion.

Die Umwandlung erfolgt nur bei Anwesenheit von Sauerstoff.

2. Schwankungen des pH-Wertes haben keinen grösseren Einfluss auf die Umwandlung. Ein Optimum der Umwandlung war bei pH $5{,}_4$—$5{,}_8$ festzustellen, ein solches der Urochrom-A-Bildung etwa bei pH $6{,}_6$.

3. Durch Zusatz von Ferrizitrat, in geringerem Grade auch von Ferrochlorid und Kupferazetat, lässt sich die Umwandlung steigern. (Betreffs des Indols erwiesen sich jedoch alle Katalysatoren gleich wirksam.)

4. Der Verlauf der Umwandlung ist folgender: zunächst findet eine reversible und fast unmittelbar darauf eine irreversible Oxydation der Askorbinsäure statt, danach eine Umwandlung der zyklischen Verbindung, sowie schliesslich eine Farbstoffbildung.

5. Die Umwandlung einer zyklischen Verbindung kann durch Zusatz einer anderen gehemmt werden. Zwar werden beide umgewandelt, doch tritt eine wechselnd starke Affinität der einzelnen Verbindungen zum Umwandlungsprozess in Erscheinung.

6. Eine Hemmung der Umwandlung von Sulfanilamid kann durch Cyren B, Östron und Thyroxin bewirkt werden, während Prolan die Umwandlung eher verstärkt.

7. Die Sulfanilamidumwandlung bleibt sich prozentual gleich in Konzentrationen, die unter physiologischen Verhältnissen vorkommen können, wie bei den Konzentrationen, mit denen gewöhnlich in den übrigen Versuchen in vitro gearbeitet wurde.

8. In sämtlichen Fällen konnte festgestellt werden, dass mit der Umwandlung eine Bildung von Urochrom A erfolgte, die aber nicht in absoluter Proportionalität zur Umwandlung stand. Das Urochrom A liess sich aus den Versuchslösungen isolieren.

Besprechung der Befunde.

Die in der Zusammenfassung aufgeführten Ergebnisse gelten für Versuche mit Salizylsäure, Sulfanilamid und Indol. Von der Einwirkung der untersuchten Katalysatoren abgesehen, sind gleichsinnige Ergebnisse mit sämtlichen untersuchten zyklischen Verbindungen erzielt worden, weshalb man annehmen dürfte, dass der Umwandlung in allen diesen Fällen ein und derselbe chemische Vorgang zugrunde liegt.

In der Schrifttumsübersicht (S. 24—26 und 33—34) wurden mehrere Untersuchungen angeführt, zu denen die hier vorgelegten Parallelen darstellen. In erster Linie seien genannt die Untersuchungen von Edlbacher und v. Segesser, Abderhalden, Holtz sowie von Leibowitz und Guggenheimer. Ich habe sowohl in den Kolbenversuchen nach Edlbacher und v. Segesser als in den Schalenversuchen nach dem Verfahren der übrigen Autoren einheitliche Ergebnisse erzielt.

Diese Ergebnisse stimmen im grossen ganzen mit den von den vorgenannten Autoren gewonnenen überein. Eine Farbstoffbildung, die also hier als eine Urochrom-A-Bildung angesehen wird, fanden Edlbacher und Holtz bei Histidinversuchen, Abderhalden bei Versuchen mit Histidin, Thyrosin, Phenylalanin und Tryptophan, in geringerem Grade jedoch nur mit aliphatischen Aminosäuren. Gemeinsam ist ferner die Feststellung, dass für die Umwandlung Sauerstoffzufuhr eine Voraussetzung ist.

In den zitierten Arbeiten wurde die Umwandlung dadurch erwiesen, dass eine Desaminierung der untersuchten Aminosäuren beobachtet wurde, oder dadurch, dass eine Entgiftung des Phenols festzustellen war. Hier ist die Umwandlung jedoch

durch quantitative Bestimmung der untersuchten Stoffe nachgewiesen worden, wodurch gezeigt werden konnte, dass die Konzentration der fraglichen Stoffe mit fortschreitendem Versuch abnahm. Von wesentlicher Bedeutung scheinen frühere Untersuchungen (EKMAN 1942) zu sein, bei denen die Umwandlung des Histidins und Tryptophans teils aus der beobachteten Desaminierung errechnet, teils direkt durch quantitative Bestimmung der beiden Aminosäuren dargetan wurde. Es zeigte sich dabei, dass nach der Desaminierung zu urteilen nur 15—20 % des Histidins und etwa 20 % des Tryptophans umgewandelt worden waren, während die quantitative Bestimmung sowohl betreffs des Tryptophans als auch des Histidins ergab, dass tatsächlich 80 % umgewandelt waren. Die quantitativen Bestimmungsmethoden gründeten sich in beiden Fällen auf Farbreaktionen des Imidazol- bzw. Indolringes, weshalb man also vermuten dürfte, dass die Umwandlung auch andere Veränderungen als eine Desaminierung einschliesst. Dies ist wichtig, da die Untersuchungen hier auf zyklische Verbindungen ausgedehnt worden sind, bei denen eine Desaminierung nicht in Frage kommen kann, wobei es aber den Anschein hat, als ob trotzdem ein und dieselbe chemische Reaktion der Umwandlung zugrunde läge.

EDLBACHER fand keine Desaminierung durch Askorbinsäure allein, sondern hielt die Anwesenheit von Eisen für notwendig. Sowohl ABDERHALDEN als HOLTZ beobachteten indessen ebenso wie ich eine Umwandlung auch mit Askorbinsäure allein. (In meinen Versuchen konnte keine katalytische Wirkung der zugesetzten Metalle beobachtet werden, ehe eine gewisse, nicht zu kleine Konzentration erreicht worden war. Dies scheint so gedeutet werden zu können, dass die Umwandlung nicht durch Spuren von Kupfer oder Eisen der Versuchslösungen katalysiert worden ist, aber die Möglichkeit bleibt natürlich, dass der Verlauf durch Spuren anderer schwerer Metalle katalysiert worden ist. Eine Schwermetallkatalyse scheint durch Zusatz von z. B. Gelatina oder Diäthyldithiocarbamid ausgeschaltet werden zu können und fortgesetzte Untersuchungen dürften dieses Problem lösen können.)

EDLBACHER fand teils, dass das optimale pH für die Histidin-

umwandlung 7,0 sei, während das Optimum für die von mir untersuchten Verbindungen bei 5,4—5,8 lag, teils beobachtete er eine bedeutend stärkere Einwirkung von pH-Schwankungen, als ich sie habe feststellen können. Dies dürfte darauf zurückzuführen sein, dass eine Aminosäure abhängiger vom pH ist, als z. B. Sulfanilamid.

Die Durchführung der Schalenversuche ist der Versuchseinrichtung vergleichbar, die bei dem Nachweis einer Entgiftung verschiedener Toxine oder z. B. von Salvarsan durch Askorbinsäure angewandt worden ist. Man dürfte annehmen können, dass es derselbe chemische Ablauf ist, der hier studiert wird. So fanden JUNGEBLUT und ZWEMER (S. 32), dass es für eine Entgiftung einer bestimmten Menge Diphtherietoxin eine optimale Askorbinsäurekonzentration gibt. Sowohl bei zu grossen als auch bei zu kleinen Askorbinsäuremengen trat eine Hemmung ein. Ferner lassen sich SIGAL und KINGS (S. 32) negative Ergebnisse bei dem Versuch, Diphtherietoxin zu entgiften, hier anführen. Nach meinen Versuchen ist die Voraussetzung für eine Umwandlung, dass die Askorbinsäure oxydiert wird, was nach den genannten Autoren in ihren Versuchen nicht stattfand.

In der einschlägigen Literatur finden sich Angaben über einen Antagonismus zwischen gewissen Geschlechtshormonen sowie Thyroxin und Askorbinsäure in vivo. Hier ist in vitro gezeigt worden, dass die Umwandlung des Sulfanilamids durch Askorbinsäure gehemmt wird, wenn man Östron, Cyren B und Thyroxin zusetzt. Analog zu den Konkurrenzversuchen, in denen gezeigt wurde, dass mehr als eine zyklische Verbindung zugleich durch Askorbinsäure umgewandelt werden kann, dürfte es wahrscheinlich sein, dass die untersuchten Hormone ebenfalls von der Askorbinsäure angegriffen worden sind. Es würde sich damit die früher gemachte Annahme (S. 42) bestätigen, dass die Hemmung einer Hormonwirkung durch Askorbinsäure in einem In-vivo-Versuch darauf beruhen kann, dass das Hormon eine Umwandlung erfahren hat.

Es sei daran erinnert, dass der Antagonismus zwischen Sexualhormonen und Askorbinsäure auch durch den auf S. 91 referierten Befund, dass Ratten während der Brunst eine erhöhte Ausscheidung von Histidin und Indoxylschwefelsäure zeigen,

demonstriert wird. Dass die Askorbinsäure an diesem Vorgang beteiligt ist, scheint durch PILLAYS (s. S. 31) Beobachtung, dass während der Ovolution Frauen eine verminderte Askorbinsäureausscheidung aufweisen, bestätigt zu werden.

In eigenen früheren Versuchen (EKMAN 1942) ist gezeigt worden, dass die Desaminierung von Histidin durch Askorbinsäure mittels eines Zusatzes von Prolan gehemmt werden konnte, und es wurde auf die Übereinstimmung dieses Befundes mit der gesteigerten Histidinausscheidung im Harn bei der erhöhten Prolanproduktion der Schwangeren hingewiesen. Man könnte erwarten, dass sich eine solche Hemmung durch das Prolan auch bei der Umwandlung anderer zyklischer Verbindungen durch Askorbinsäure nachweisen lassen müsste. Das war indessen nicht der Fall, vielmehr trat bei Zusatz grosser Mengen Prolan eine verstärkte Umwandlung ein. Die bisher ausgeführten Versuche vermögen keine Erklärung für diese widersprechenden Resultate zu geben.

In der Schrifttumsübersicht wurden Untersuchungen genannt, die gezeigt haben, dass eine Farbstoffbildung sowohl in vivo als in vitro durch Askorbinsäure gehemmt worden ist. Im Einklang damit haben die oben geschilderten In-vitro-Versuche der vorliegenden Arbeit ergeben, dass »die totale Farbstoffbildung» (s. S. 90) bei einem Überschuss von Askorbinsäure gehemmt wird. Dagegen konnte unter den vorliegenden Versuchsbedingungen keine Hemmung der Urochrom-A-Bildung beobachtet werden.

Versucht man aus den gewonnenen Ergebnissen Rückschlüsse auf die Art der beobachteten Umwandlung zu ziehen, so muss betont werden, dass entgegen der Anschauung LEIBOWITZ' und GUGGENHEIMERS keine Paarung der Askorbinsäure mit der zyklischen Verbindung nachgewiesen worden ist.

Ohne Zufuhr von Sauerstoff findet keine Umwandlung statt. Diese muss daher einen oxydativen Vorgang einschliessen. Es findet auch eine Oxydation der Askorbinsäure statt. Indessen lassen sich gewisse Anhaltspunkte dafür nennen, dass auch der Verlauf im übrigen als eine Oxydation aufgefasst werden könnte. Der chemische Verlauf liess sich in den Versuchen, in denen der zeitliche Ablauf der Umwandlung studiert wurde, in drei Prozesse aufteilen, die aufeinander folgten, nämlich die Askorbinsäure-

oxydation, die Umwandlung der zyklischen Verbindung und die Farbstoffbildung. (Dass die Farbstoffbildung ein Prozess für sich war, zeigte sich auch auf andere Weise: es liessen sich unterschiedliche pH-Optima für Umwandlung und Farbstoffbildung unterscheiden, und die Parallelität zwischen diesen beiden war nicht vollständig.) Möglicherweise dürfte es sich bei sämtlichen Prozessen um Oxydationen handeln. Einen Beleg dafür, dass die Farbstoffbildung ein oxydativer Vorgang ist, kann man darin sehen, dass sie durch Askorbinsäure im Überschuss gehemmt wird. Die Askorbinsäure tritt dann nämlich wahrscheinlich als ein Reduktionsmittel auf, im Einklang mit Szent-Györgyis Anschauung (siehe S. 23).

Abderhaldens Nachweis der Entstehung von Aldehyden bei der Umwandlung von Aminosäuren sowie Holtz' Theorie, dass die Askorbinsäurewirkung auf der Entstehung von Peroxyden beruht, bieten weitere Stützen für die Annahme, dass es sich bei der beobachteten Umwandlung um einen oxydativen Prozess handelt.

II. In vivo.

Im vorigen Abschnitt wurde mit mehreren Beispielen gezeigt, dass in vitro eine Umwandlung zyklischer Verbindungen durch Einwirkung von Vitamin C vor sich gehen kann, wobei sich Farbstoffe bilden, die mit einer Urochrom-A-Bestimmungsmethode quantitativ bestimmt werden können und als Urochrom A isoliert werden können. Diese Umwandlung fand auch bei Konzentrationen der reagierenden Stoffe statt, wie sie im lebenden Organismus vorkommen können.

In früheren Arbeiten (Ekman 1941 und 1942) wurde gezeigt, dass eine Umwandlung von Histidin und Phenol durch Askorbinsäure auch in vivo erfolgt, und um diese Befunde zu bestätigen, sind auch Untersuchungen mit anderen zyklischen Verbindungen vorgenommen worden.

Findet eine Umwandlung einer zugeführten zyklischen Verbindung durch Askorbinsäure auch in vivo statt, so muss man dies dadurch zeigen können, dass die Ausscheidung der zyklischen Verbindung bei C-Vitaminmangel zunimmt und bei über-

schüssigem Vitamin C abnimmt. Steigert man andererseits die Einverleibung zyklischer Verbindungen, so kann sich vielleicht ein erhöhter Verbrauch von Vitamin C durch eine verminderte Askorbinsäureausscheidung nachweisen lassen. Da die Umwandlung in vitro unter Bildung von Urochrom A vor sich geht, muss man auch in vivo eine gesteigerte Ausscheidung dieser Farbstoffe bei vermehrter Zufuhr zyklischer Verbindungen beobachten können.

Schliesslich muss sich durch Einverleibung einer zyklischen Verbindung die Umwandlung einer anderen hemmen lassen, was in gesteigerter Ausscheidung dieser Verbindung zum Ausdruck kommen muss.

Es sind hier die gleichen Stoffe untersucht worden wie bei den Versuchen in vitro, doch habe ich ausserdem Gelegenheit gehabt, gewisse Untersuchungen an benzolexponierten Arbeitern vorzunehmen.

1. Die Ausscheidung zyklischer Verbindungen mit dem Harn bei wechselnder Zufuhr von Vitamin C.

a. *Die Ausscheidung von Indoxyl und Indol.*

Allgemein gilt das Tryptophan der Kost als die Muttersubstanz der im Harn vorkommenden Indoxylschwefelsäure und Indoxylglykuronsäure. Die Dekomposition vollzieht sich im Darm unter Einwirkung verschiedener Bakterien, und Schwankungen in der Ausscheidung von Indoxylverbindungen hat man in der Hauptsache als das Ergebnis von Veränderungen in der Bakterienflora des Darmes aufgefasst.

Das Bakterienprodukt ist Indol, und die Oxydation zu Indoxyl und die Paarung mit einer Säure finden nach der Resorption und mit aller Wahrscheinlichkeit in der Leber statt. Der Organismus scheint die Fähigkeit zu besitzen, auf diese Weise alles zugeführte Indol umzuwandeln, und normalerweise soll keine Ausscheidung mit dem Harn vorkommen.

Zwar gab Jaffé 1908 an, dass man durch Destillation des Harns freies Indol nachweisen könne. Vaughan (1932) untersuchte dagegen 147 Harne von normalen und kranken Meschen, ohne Indol in irgendeinem Harn zu finden — die untere Grenze der mit seinem Verfahren fassbaren Menge dürfte etwa bei 2 mg pro Tag gelegen haben.

Mit ihrer (auf S. 64 referierten) bedeutend empfindlicheren quantitativen Methode konnten indessen Forbes und Neale (1934) zeigen, dass kleine Indolmengen, gewöhnlich 0,5—1,5 mg pro Tag, in den meisten

Fällen von Polyarthritis, Schwangerschaftstoxikose, Pellagra, Tuberkulose und oft auch bei Diabetes ausgeschieden wurden.

BORTOLUCCI (1938) konnte mit Hilfe des FORBES-NEALEschen Verfahrens Indolurie bei Schwangeren feststellen.

Nach Erscheinen der Arbeit von FORBES und NEALE griff VAUGHAN 1936 das Problem erneut an und meinte zeigen zu können, dass wahllos herausgegriffene Harne, die man stehen lässt, nach etwa einem Tage freies Indol enthalten. Nach VAUGHANS Meinung kommt das daher, dass Bakterien im Harn aus einem Precursor — wahrscheinlich Tryptophan — Indol freimachen.

Durch den Nachweis von Indol mit Hilfe des FORBES-NEALEschen Verfahrens nach Zusatz von Tryptophan und Bakterien zu indolfreiem Harn konnten CARNES und LEWIS (1938) die VAUGHANsche Ansicht bestätigen.

Eine entscheidende Kritik der von FORBES und NEALE gewonnenen Ergebnisse bedeutet das jedoch nicht. Bloss erscheint unsicher, ob bei den von FORBES und NEALE angegebenen Krankheitszuständen Indol oder ein Precursor ausgeschieden wird. Wahrscheinlich ist, dass der Indolumsatz unter gewissen Verhältnissen Störungen aufweisen kann.

An experimentellen Arbeiten sind in diesem Zusammenhang die Forschungen von HOUSSAY, DEULOFEU und MAZZOCCO (1935) zu nennen, die dartaten, dass von zugeführtem Indol nur etwa 1/3 als Indoxyl ausgeschieden wurde. In ähnlichen Versuchen fanden PINELLI und PUGIONI (1934), dass 50 % als Indoxyl ausgeschieden wurden. Keiner von ihnen hat kontrolliert, ob Indol ausgeschieden wurde.

Dass die Indoxylausscheidung nicht ausschliesslich durch die Bakterienflora des Darmes bedingt ist, zeigen die Untersuchungen von SANADA (1937): eine erhöhte Indoxylausscheidung wurde erzielt bei Einverleibung von Thyroxin, Ovarialhormon und Hodenhormon, eine verminderte bei Einverleibung von Dijodthyrosin und Prolan.

In erster Linie wurde die Ausscheidung von Indoxyl im Harn von Meerschweinchen untersucht, die bloss die Grundkost (S. 92) bekamen. Abb. 10 lässt eine gewisse Tendenz der Indoxylausscheidung erkennen, sich entsprechend der Askorbinsäurezufuhr zu ändern. Bei Einverleibung von Askorbinsäure sinkt die Indoxylausscheidung, gibt man weniger Askorbinsäure, so nimmt die Ausscheidung von Indoxyl zu. In den Endstadien des Skorbutversuchs findet man bei den Versuchstieren eine weit höhere Ausscheidung als bei den Kontrolltieren.

Im grossen ganzen zeigen jedoch sämtliche Tiere eine im Verlauf des Versuchs fortschreitend sinkende Indoxylausscheidung. Vor dem Versuch bekamen die Tiere ein freies Futter (Rüben, Körner), von dem sich die Kost während des Versuchs erheblich

unterscheidet, und wahrscheinlich hat man hierin die Erklärung für die Abnahme der Indoxylausscheidung zu sehen. Die gleiche Erfahrung wurde auch an anderen Meerschweinchenreihen gemacht.

Man hätte indessen einen grösseren Unterschied zwischen der Indoxylausscheidung der Skorbuttiere und derjenigen der Kontrolltiere während des ganzen Skorbutversuchs erwartet. Die besonders in der Mitte des Skorbutversuchs geringe Indoxyl-

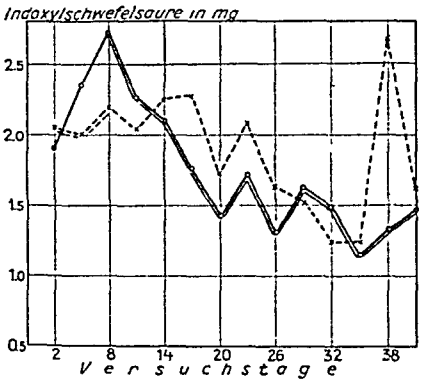

Abb. 10. *Die Ausscheidung von Indoxylschwefelsäure bei wechselnden Askorbinsäuregaben.*

Der Versuch umfasst zwei Meerschweinchengruppen von je 5 Tieren. Einfach gezeichnete Kurve: Skorbutkost. Doppelte Kurve: Täglich 100 mg Askorbinsäure durch Injektion. Die Indoxylausscheidung ist als mg/Tag angegeben, für ein Körpergewicht von 200 g berechnet.

ausscheidung der Skorbuttiere lässt sich möglicherweise so erklären, dass, da das Ausgangsmaterial der Indoxylbildung der Tryptophangehalt der Nahrung sein dürfte, durch die im Laufe des Skorbutversuchs immer mehr abnehmende Nahrungsaufnahme der Tiere das Auftreten einer gesteigerten Indoxylausscheidung verhindert wird. (Um diesen Einfluss der Nahrungsaufnahme zu kompensieren, ist in Abb. 10 die Indoxylausscheidung für ein Meerschweinchen von 200 g Gewicht berechnet worden.)

Um die Verhältnisse besser studieren zu können, wurde der Versuch folgendermassen wiederholt: die Indoxylausscheidung wurde dadurch standardisiert, dass sämtliche Tiere täglich 20 mg Indol eingespritzt bekamen. Hierdurch stieg die Indoxylaus-

scheidung so erheblich, dass Schwankungen in der Nahrungsaufnahme keine Rolle spielen konnten, und gleichzeitig wurde ein etwaiger Einfluss von seiten der Bakterienflora des Darmes ausgeschaltet.

Wie aus Abb. 11 a und b ersichtlich, lieferten die so durchgeführten Versuche ein überzeugenderes Resultat: die Indoxyl-

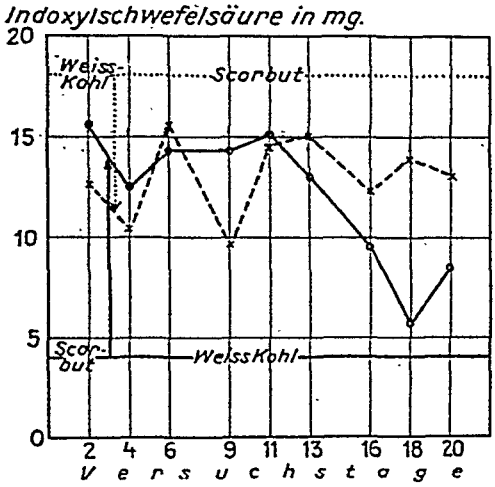

Abb. 11 a.

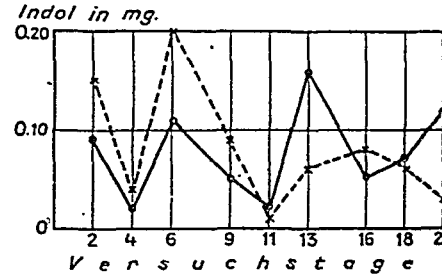

Abb. 11 b.

Abb. 11 a. *Die Ausscheidung von Indoxylschwefelsäure bei täglicher Einverleibung von 20 mg Indol und wechselnden Askorbinsäuregaben.*

Abb. 11 b. *Wie 11 a, doch wird hier die Ausscheidung an freiem Indol angegeben (in mg/Tag pro Versuchstier).*

Der Versuch umfasst zwei Meerschweinchengruppen von 4 und 6 (abgebrochene Kurve) Tieren. Die Kontrolltiere bekamen täglich 10 g Weisskohl; an den Versuchstagen 17—20 ausserdem 1/4 Zitrone pro Tier. Überlebende Skorbuttiere: nach 13 Tagen sämtl., nach 16 Tagen 4, nach 16 Tagen 3 und nach 18 Tagen 2 Tiere.

ausscheidung der Skorbuttiere zeigt eine steigende Tendenz und liegt vor allem die ganze Zeit auf hohem Niveau. Die Kontrolltiere, die zu Beginn des Versuches Skorbutkost bekamen, zeigen mit steigender C-Vitaminzufuhr eine stetig abnehmende Indoxylausscheidung.

Während des ganzen Versuchs wurde gleichzeitig die Ausscheidung freien Indols im Harn studiert. Die ausgeschiedene

Menge betrug indessen die ganze Zeit durchschnittlich 0,1 mg. Die Meerschweinchen können also, unabhängig von der C-Vitaminzufuhr, durch Oxydation zu Indoxyl oder auf andere Weise die einverleibten Indolmengen (20 mg/Tag) umwandeln.

Die Versuche dürften zeigen, dass die Indoxylausscheidung bei Skorbut ansteigt, aber auf normale Höhe zurückgeführt und darauf gehalten werden kann, wenn man Askorbinsäure einverleibt oder an Vitamin C reiche Vegetabilien füttert. Es erschien nun wünschenswert zu untersuchen, ob auch die normale Indoxylausscheidung durch stark veränderte Askorbinsäurezufuhr beeinflusst werden könnte. Dies gelang indessen nicht. Vielmehr war bei hohen Askorbinsäuregaben eher eine gesteigerte Indoxylausscheidung festzustellen.

b. *Ausscheidung von Salizylsäure.*

In Kap. II wurde erörtert, unter welchen Formen die Salizylsäure ausgeschieden wird. In Selbstversuchen wurde gezeigt, dass bei einer Zufuhr von 0,5 g pro Tag die Salizylsäure quantitativ ausgeschieden wird.

An Untersuchungen über die einschlägigen Verhältnisse im Tierversuch sei die Arbeit von Blume und Nohara (1933) erwähnt. Sie fanden bei Kaninchen, die mit Rüben gefüttert wurden, etwa 80 % der einverleibten Salizylsäuremenge wieder, bei Fütterung mit Grünfutter nur etwa 50 %. In einer späteren Arbeit gibt Blume (1935) die Ausscheidung mit 80 % an.

Die Salizylsäureausscheidung wurde an Meerschweinchen studiert, die 50 mg Natriumsalizylat pro Tag einverleibt bekamen. Bestimmt wurde die Gesamtsalizylsäure (freie + gebundene), und die Ausscheidungswerte wurden als Natriumsalizylat berechnet.

Normalerweise wird etwa die Hälfte der einverleibten Menge ausgeschieden (Abb. 12).

Füttert man die Tiere mit Skorbutkost, so steigt die Ausscheidung erheblich und kann dann durch Darreichung askorbinsäurereicher Vegetabilien (Weisskohl) wieder zum Sinken gebracht werden. Die Kontrolltiere, die nur Askorbinsäure erhielten, hatten die ganze Zeit eine niedrige Salizylsäureausscheidung. Eine Steigerung der Askorbinsäurezufuhr bewirkt indessen keine weitere Senkung der Ausscheidung, vielmehr tritt

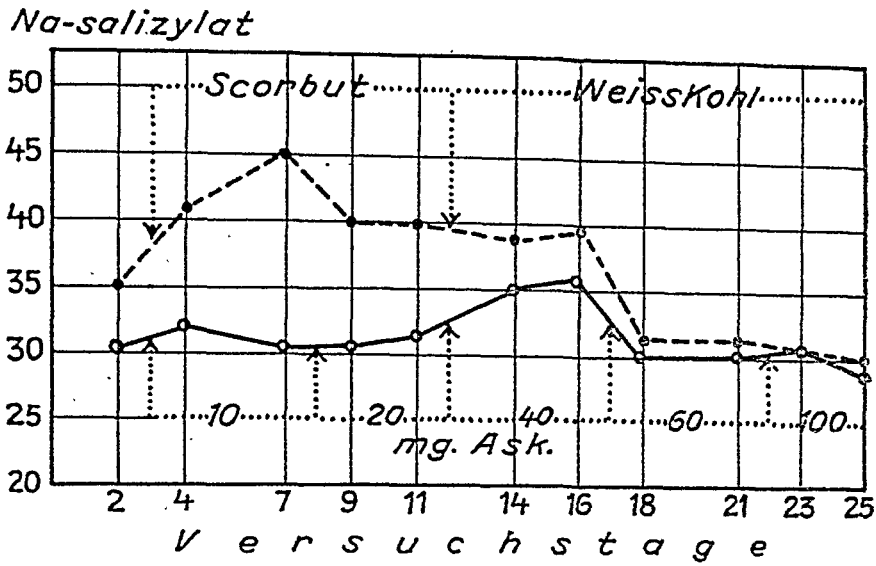

Abb. 12. *Die Ausscheidung von Salizylsäure im Harn bei wechselnden C-Vitamingaben.*

Der Versuch umfasst zwei Meerschweinchengruppen von 6 (Skorbutgruppe) bzw. 5 Tieren. Sämtl. Tiere bekamen täglich 50 mg Natriumsalizylat per os. Die Askorbinsäure wurde eingespritzt. Weisskohl wurde in Tagesmengen von 10 g gereicht, etwa 10 mg Askorbinsäure entsprechend. Der Harn wurde in Zweitagesperioden gesammelt.

bei einer täglichen Askorbinsäuregabe von 40 mg eine vorübergehende Steigerung der Salizylsäureausscheidung ein.

Ein Vergleich zwischen der Ausscheidung von freier und gebundener Salizylsäure bei wechselnder Zufuhr von Vitamin C ergab, dass das Verhältnis von freier und gebundener Salizylsäure zueinander die ganze Zeit konstant blieb. Die C-Vitaminzufuhr hat also nichts mit der Koppelung der Salizylsäure zu tun.

c. *Ausscheidung von Sulfanilamid.*

Sulfanilamid wird im Harn teils frei, teils in gekoppelter Form ausgeschieden. Soweit ich habe finden können, liegen keine Untersuchungen über die Ausscheidung von Sulfanilamid im Meerschweinchenharn vor. Dagegen hat z. B. SIMESEN (1940) die Ausscheidung bei der Ratte untersucht und etwa 45 % der einverleibten Menge im Harn wiedergefunden. Am Menschen sind sehr viele einschlägige Untersuchungen gemacht worden; SIMESEN (1940 a) z. B. fand 50—70 % im Harn, während STEWART

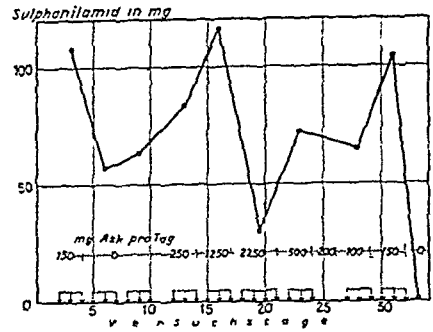

Abb. 13. *Die Sulfanilamidumwandlung bei Tagesgaben von 1 g Sulfanilamid und wechselnden Askorbinsäuregaben.* (Selbstversuch.)

Die Punkte der Kurve entsprechen den Mittelwerten von Versuchsperioden mit einer Dauer von gewöhnlich 3 Tagen. (Die Tage der Sulfanilamidbestimmungen sind durch Pfeile kenntlich gemacht; die Versuchstage innerhalb einer Versuchsperiode sind durch einen Querstrich gekennzeichnet. Bestimmung von Gesamtsulfanilamid.)

et al. (1938) bedeutend höhere Werte fanden, nämlich 88—98 %. Im allgemeinen dürfte man annehmen, dass peroral zugeführtes Sulfanilamid quantitativ aus dem Darm resorbiert wird.

Die Sulfanilamidausscheidung ist hier teils in Selbstversuchen, teils in Meerschweinchenversuchen studiert worden.

Im Selbstversuch nahm ich täglich peroral 1000 mg Sulfanilamid (um 13 Uhr). Auch die Askorbinsäure wurde per os eingenommen, doch in drei Portionen (um 9, 17 und 21 Uhr). Der grösste Teil des eingenommenen Sulfanilamids, 85—100 %, wurde im Harn wiedergefunden, und um die Schwankungen in der Ausscheidung leichter graphisch darstellen zu können, ist in Abb. 13 nicht die ausgeschiedene Menge, sondern der Unterschied zwischen der einverleibten und der ausgeschiedenen Menge angegeben. Dieser Unterschied, also die nicht ausgeschiedene Menge, ist als im Körper verbrannt betrachtet worden.

Die drei Höhepunkte der Kurve in Abb. 13 (die also eine maximale Verbrennung des einverleibten Sulfanilamids bezeichnen), fallen alle mit einer gesteigerten Einverleibung von Vitamin C zusammen. Die Tiefpunkte der Kurve fallen in zwei Fällen mit dem Streichen der zusätzlichen Vitamin-C-Gaben zusammen,

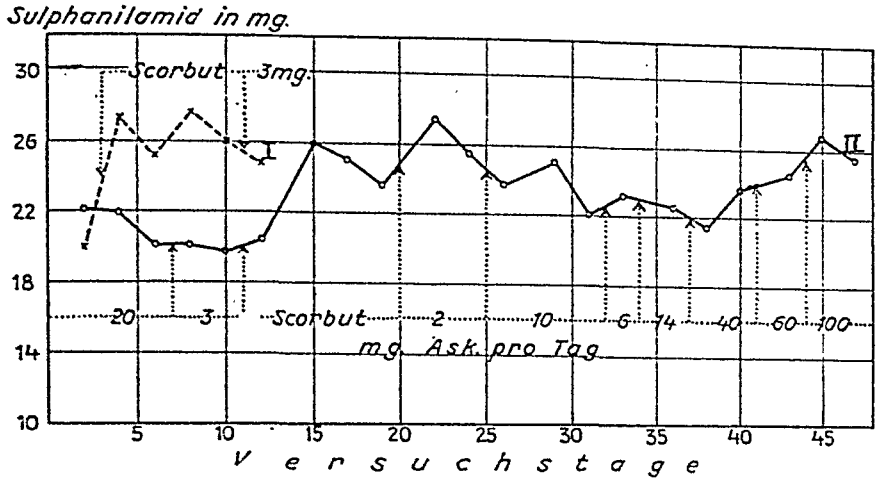

Abb. 14. *Die Sulfanilamidausscheidung bei wechselnden Askorbinsäuregaben.*

Der Versuch umfasst zwei Meerschweinchengruppen von je 5 Tieren. Bei Gruppe I wurde der Versuch abgebrochen, als die Sulfanilamidausscheidung nach Einverleibung von Vitamin C eine fallende Tendenz verriet.

Sämtl. Tiere bekamen täglich 50 mg Sulfanilamid per os. Die Askorbinsäure wurde eingespritzt. Der Harn wurde in Zweitagesperioden gesammelt. Bestimmung von Gesamtsulfanilamid.

Ein Versuchstier der Gruppe II starb nach 24 Tagen.

in einem Falle aber mit einer ausnehmend hohen C-Vitamingabe. Es scheint also eine gewisse, nicht übermässige Askorbinsäurezufuhr für die Sulfanilamidverbrennung optimal zu sein, und ferner scheint diese letztere sowohl durch Vitamin-C-Mangel als durch sehr hohe Überschüsse an Vitamin C gehemmt werden zu können.

Der Versuch wurde an Meerschweinchen wiederholt, und wie aus Abb. 14 hervorgeht, mit ähnlichem Ergebnis. Bei Skorbutkost steigt die Ausscheidung (nimmt die Verbrennung im Organismus ab) und kann dann durch Einverleibung mässiger Askorbinsäuremengen wieder auf den normalen Stand gebracht werden. Werden grosse Mengen Askorbinsäure gereicht, so steigt die Ausscheidung wieder.

Sowohl im Meerschweinchenversuch als im Selbstversuch konnte festgestellt werden, dass das Verhältnis zwischen freiem

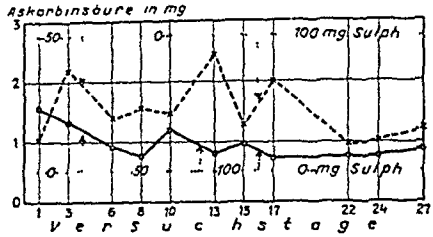

Abb. 15. *Die Einverleibung von Sulfanilamid ist ohne Einfluss auf die Ausscheidung von Askorbinsäure mit dem Harn.*

Der Versuch umfasst zwei Gruppen von je 5 Tieren. Sämtl. Tiere bekamen täglich 20 mg Askorbinsäure eingespritzt. Sulfanilamid per os. Askorbinsäurebestimmung nach TAUBER nach vorheriger Schwefelwasserstoffbehandlung (S. 78).

und gebundenem Sulfanilamid in der ausgeschiedenen Menge konstant ist, dass also die Koppelung nicht durch die C-Vitaminzufuhr beeinflusst wird. Ebenso wurde kontrolliert, dass das ausgeschiedene Sulfanilamid, während der Harn gesammelt wurde, nicht ausgefällt wurde.

2. Die Askorbinsäureausscheidung im Harn bei wechselnder Zufuhr von zyklischen Verbindungen.

a. *Zufuhr von Sulfanilamid und Natriumsalizylat.*

Ich habe es für richtig angesehen, mit einer Askorbinsäurezufuhr zu arbeiten, die nur eine geringere Ausscheidung im Harn mit sich bringt. Falls die Zufuhr zyklischer Verbindungen einen gesteigerten Askorbinsäureverbrauch im Gefolge hat, so dürfte sich dies bei einer mässigen Askorbinsäurezufuhr eher zeigen als bei einem grossen Überschuss. Die zyklische Verbindung ist dagegen in grossen Mengen einverleibt worden.

Wie Abb. 15 zeigt, wird bei einer Zufuhr von 20 mg Askorbinsäure und 50—100 mg Sulfanilamid pro Tag die Askorbinsäureausscheidung nicht durch das Sulfanilamid beeinflusst. In einem anderen Versuch konnte gezeigt werden dass tägliche Gaben von 150 mg Natriumsalizylat bei gleichzeitiger Zufuhr von 20 mg

Askorbinsäure keine Einwirkung auf die Askorbinsäureausscheidung ausübten.

Ähnliche Ergebnisse wurden in Versuchen mit denselben zyklischen Verbindungen, doch mit täglichen Askorbinsäuregaben von 60—100 mg gewonnen. Auch Phenol konnte die Askorbinsäureausscheidung nicht beeinflussen.

b. *Zufuhr von Benzol.*

Durch das Entgegenkommen von Prof. S. Forssman an der Berufshygienischen Abteilung des Staatl. Instituts für Volksgesundheit, Stockholm, hatte ich Gelegenheit, an einer Untersuchung teilzunehmen, die über gewisse Verhältnisse bei benzolexponierten Arbeitern Klarheit schaffen sollte (Forssman und Frykholm 1943). Angaben über das Versuchsmaterial und gewisse Ergebnisse Forssmans und Frykholms zitiere ich mit Erlaubnis der Verfasser nach ihrem Manuskript:

»Methodik: In einer Tiefdruckanstalt wurden sämtliche 26 Arbeiter untersucht, die unter gleichen Arbeitsverhältnissen dem Einfluss von Benzoldämpfen ausgesetzt waren. Diese wurden mit 23 Arbeitern desselben Unternehmens verglichen, die weder beruflich noch sonst mit Benzol in Berührung kamen. Wir erhielten sämtliche Angaben über bisherige Dauer der Anstellung, Lebensgewohnheiten, Ernährung und frühere Krankheiten sowie etwaige Beschwerden bei der Arbeit. Die Benzolexponierten wurden gleichzeitig klinisch untersucht, der Blutstatus wurde aufgenommen usw.; die diesbezüglichen Ergebnisse siehe bei Oldfelt (1943). Die Arbeiter lieferten täglich den Morgenharn ab, ausser denen, die in der Nachmittagsschicht arbeiteten und die den Harn unmittelbar vor Beginn der Arbeit liessen. Der Harn wurde uns in dunklen verschlossenen Flaschen von 200 ml binnen wenigen Stunden zugeschickt.«

In diesen Harnproben wurden der C-Vitamingehalt, das Sulfat (freies und Gesamtsulfat) und die Muconsäure von Forssman und Frykholm in Stockholm bestimmt.

Der Belastungsversuch wurde folgendermassen ausgeführt: Die Askorbinsäureausscheidung wurde einige Tage lang verfolgt, dann wurden am ersten Tage 600 mg Askorbinsäure (Tabletten) und anschliessend täglich 300 mg verabfolgt, bis in wenigstens zwei Harnproben (Morgenharn) eine Askorbinsäurekonzentration von 5—10 mg% vorlag. Forssman und Frykholm fassen die

Ergebnisse wie folgt zusammen: »Wenn man diejenigen als gesättigt betrachtet, die eine Konzentration von 7 mg% im Harn erreicht hatten» ... zeigt es sich ... »dass von dem Normalmaterial 19 von 23, also 83 %, binnen 10 Tagen gesättigt waren. Von den benzolexponierten Arbeitern sind in der gleichen Zeit nur 10 von 26 gesättigt, also 38 %. Hier liegt ein deutlicher und sicherer Unterschied zwischen den beiden Untersuchungsgruppen vor. Mehrere der Benzolexponierten erreichen nie die Konzentration von 5 mg% Askorbinsäure im Harn, trotz Tagesgaben von 300 mg während einer Zeit von 23 Tagen.»

Bemerkenswert ist, dass die benzolexponierten Arbeiter vor der Verabfolgung der C-Vitamintabletten eine etwas höhere Askorbinsäureausscheidung zeigten als das Kontrollmaterial ($2{,}_{20}$ bzw. $1{,}_{58}$ mg%). Die Askorbinsäure wurde im Prinzip nach dem auf S. 78 beschriebenen Titrationsverfahren nach TILLMANS bestimmt. Wie im folgenden gezeigt werden wird, war indessen die Urochrom-A-Ausscheidung gerade bei den Benzolexponierten erhöht, und die erhöhten Askorbinsäurewerte dürften also durch Urochrom A und nicht durch Vitamin C bedingt gewesen sein, gemäss den auf S. 83 vorgelegten Ergebnissen.

Die Untersuchungen von FORSSMAN und FRYKHOLM zeigen, dass Personen, die der Einwirkung von Benzol ausgesetzt sind, schwerer mit Vitamin C gesättigt werden können als andere. Nach der allgemeinen Anschauung liesse sich dieses grössere Defizit bei den Benzolbeeinflussten als die Folge eines durch das Benzol verursachten grösseren C-Vitaminbedarfs deuten.

3. Die Urochrom-A-Ausscheidung im Harn bei Zufuhr von zyklischen Verbindungen.

a. *Zufuhr von Salizylsäure, Sulfanilamid und Indol.*

Aus Abb. 16—18 geht hervor, dass die Einverleibung von Salizylsäure, Sulfanilamid und Indol eine gesteigerte Ausscheidung von Urochrom A mit sich bringt. Der wahrscheinliche Hergang bei der Bildung des Urochroms A wird in Kapitel IV besprochen.

Das Urochrom A wurde aus den untersuchten Harnen isoliert.

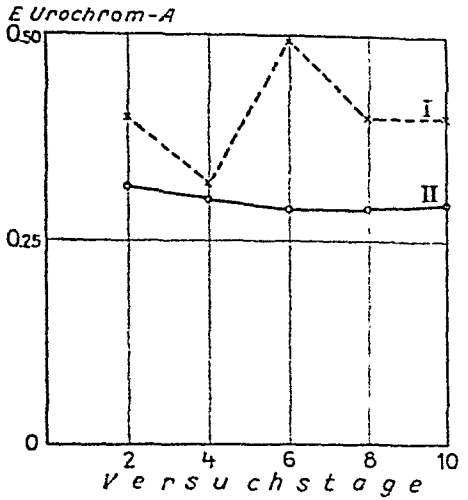

Abb. 16. *Steigerung der Urochrom-A-Ausscheidung bei Zufuhr von Natriumsalizylat.*

Der Versuch umfasst zwei Meerschweinchengruppen von je 4 Tieren. Urochrom A wird mit dem Extinktionswert bei Bestimmung in einer Tagesmenge von 25 ccm angegeben. Die Tiere in Gruppe I erhielten täglich 150 mg Natriumsalizylat per os. Tägliche Vitamin-C-Zufuhr: 20 mg Askorbinsäure durch Injektion.

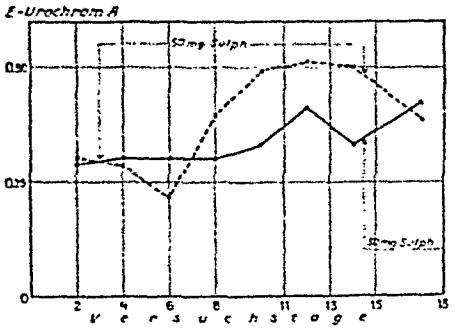

Abb. 17.

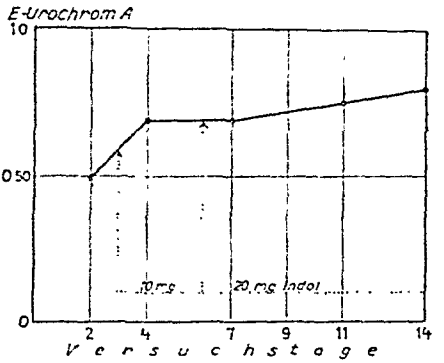

Abb. 18.

Abb. 17. *Steigerung der Urochrom-A-Ausscheidung bei Zufuhr von Sulfanilamid.*

Der Versuch umfasst zwei Meerschweinchengruppen von je 5 Tieren. Sulfanilamid wurde per os gegeben. Urochrom A wird mit dem Extinktionswert bei Bestimmung in einer Tagesmenge von 25 ccm angegeben. Tägliche Vitamin-C-Zufuhr: 20 mg Askorbinsäure durch Injektion.

Abb. 18. *Steigerung der Urochrom-A-Ausscheidung bei Zufuhr von Indol.*

Der Versuch umfasst eine Gruppe Meerschweinchen von 5 Tieren. Urochrom A wird mit dem Extinktionswert bei Bestimmung in einer Tagesmenge von 25 ccm angeben. Indol wurde durch Injektion gegeben. Tägliche Vitamin-C-Zufuhr: Weisskohl etwa 10 mg Askorbinsäure entsprechend.

TABELLE 51.

Steigerung der Urochrom-A-Ausscheidung bei Zufuhr von Benzol (Druckereiarbeiter).

Versuchseinrichtung: Siehe den nebenstehenden Text und S. 131.

	Vor der Vitamin-C-Belastung		Tage nach Beginn der Vitamin-C-Belastung			
			4		11	
	Urochr. A E	Anzahl Bestimm.	Urochr. A E	Anzahl Bestimm.	Urochr. A E	Anzahl Bestimm.
Kontrolle 22 Arbeiter	0,28	38	0,31	19	0,28	18
Benzol 21 Arbeiter	0,38	44	0,46	17	0,28	19

b. *Zufuhr von Benzol.*

Im Zusammenhang mit den auf S. 131 erwähnten Untersuchungen von Forssman und Frykholm an benzolexponierten Arbeitern wurden Harnproben in 50-ccm-Flaschen, mit einer etwa cm-dicken Toluolschicht versehen, nach Lund geschickt und hier von mir auf den Urochrom-A-Gehalt (und Polyphenolgehalt, siehe S. 147) untersucht.

Das Ergebnis der Urochrom-A-Bestimmungen ist in Tab. 51 verzeichnet. Die Benzolexponierten scheiden etwa 38 % mehr Urochrom A aus als das Vergleichsmaterial. (Bei Einverleibung von Vitamin C sinkt die Urochrom-A-Ausscheidung der Benzolexponierten.)

4. Die Histidinausscheidung im Harn bei wechselnder Zufuhr von Phenol.

Es schien von Interesse zu sein, wenn gezeigt werden könnte, dass auch bei Ratten, die selbst ihr Vitamin C synthetisieren, ähnliche Verhältnisse in bezug auf den Umsatz zyklischer Verbindungen herrschen wie beim Meerschweinchen und Menschen.

In der Schrifttumsübersicht wurden Untersuchungen beispielsweise von Longenecker et al. (S. 37) erwähnt, aus denen hervor-

TABELLE 52.

Einwirkung der Zufuhr von Phenol auf die Histidinausscheidung bei Ratten.

Der Versuch umfasst zwei Gruppen von je 6 Versuchstieren. Der Harn wurde in Zweitagesperioden gesammelt. Die Sammelperioden waren durch eine 24stündige Pause voneinander getrennt. Die Histidinausscheidung ist in mg/Tag angegeben, für ein Körpergewicht des Versuchstieres von 200 g berechnet. Sämtliche Tiere bekamen täglich 20 mg Askorbinsäure eingespritzt. Phenol durch Injektion einverleibt.

Versuchsperiode		1	2	3	4	5
Gruppe I	Phenolzufuhr mg/Versuchsperiode			25 × 1	5 × 2	
	Histidinausscheidung	0,86	1,04	1,46	0,91	0,72
Gruppe II	Phenolzufuhr mg/Versuchsperiode					5 × 3
	Histidinausscheidung	0,91	0,98	0,85	0,63	1,11

ging, dass Ratten bei Einverleibung zyklischer Verbindungen durch gesteigerte Askorbinsäureproduktion reagieren.

Wird diese erhöhte Produktion zur Umwandung der einverleibten Verbindungen verwandt, so dürfte man durch Zufuhr einer zyklischen Verbindung, trotz der kompensatorischen Steigerung der Vitamin-C-Produktion, die Ausscheidung einer anderen zyklischen Verbindung mit dem Harn beeinflussen können. Als Beispiel wurde das Studium der Histidinausscheidung bei wechselnder Phenolzufuhr gewählt. Wie aus Tab. 52 hervorgeht, gelang es, durch Zufuhr von verhältnismässig kleinen Phenolmengen die Histidinausscheidung hochgradig zu steigern.

Zusammenfassung der Versuchsergebnisse.

1. Die Ausscheidung von Indoxylschwefelsäure, Salizylsäure und Sulfanilamid im Harn lässt sich durch Zufuhr von Vitamin C beeinflussen.

Die Ausscheidung steigt bei Skorbut, fällt bei Einverleibung

mässiger Askorbinsäuremengen und steigt wieder bei Zufuhr sehr grosser Mengen Askorbinsäure.

2. Die Ausscheidung von Askorbinsäure im Meerschweinchenharn wird durch Zufuhr von Salizylsäure, Sulfanilamid und Phenol nicht beeinflusst. (Benzolexponierte Arbeiter zeigten dagegen ein grösseres C-Vitamindefizit als Kontrollpersonen [FORSSMAN und FRYKHOLM].)

3. Bei Einverleibung von Indol, Salizylsäure, Sulfanilamid und Benzol steigt die Ausscheidung von Urochrom A im Harn.

4. Bei Einverleibung von Phenol steigt die Histidinausscheidung im Harn der Ratte.

Besprechung.

In eigenen Versuchen ist gezeigt worden, dass Histidin (EKMAN 1941), Phenol (EKMAN 1942), Salizylsäure, Sulfanilamid und Indol (siehe oben) durch Vitamin C in vivo unter Bildung von Urochrom A umgewandelt werden können.

Veränderungen in der Urochrom-A-Ausscheidung und im C-Vitaminstoffwechsel bei benzolexponierten Arbeitern können darauf hindeuten, dass auch Benzol durch Vitamin C umgewandelt wird.

Die Benzolversuche sind nur am Menschen gemacht worden. Die übrigen Stoffe sind in Meerschweinchenversuchen studiert worden, die Histidin- und Sulfanilamidausscheidung auch im Selbstversuch. Phenol konnte durch Askorbinsäure auch bei Pflanzen (Kresse) entgiftet werden (EKMAN 1942).

Die Ergebnisse waren die gleichen bei peroraler Einverleibung der Askorbinsäure wie bei Injektion, weshalb die beobachtete Einwirkung auf die zyklischen Verbindungen nicht durch eine Reaktion im Magen-Darmkanal bedingt gewesen sein kann. Im Pflanzenversuch konnte durch eine besondere Vorrichtung das Zusammentreffen von Phenol und Askorbinsäure bis nach erfolgter Resorption verhindert werden.

Die hier studierte Wirkung der Askorbinsäure auf die Ausscheidung zyklischer Verbindungen könnte die indirekte Folge einer Einwirkung auf die Diurese sein. Indessen hat z. B. ABBASY (1937) zeigen können, dass Askorbinsäure eine gewisse diurese-

fördernde Wirkung besitzt. Wenn trotzdem bei mässigen Askorbinsäuregaben eine verminderte Ausscheidung der zyklischen Verbindung beobachtet wurde, kann es sich also nicht um eine Diuresewirkung gehandelt haben. Dagegen wäre eine Ausschwemmung der zyklischen Verbindung als Erklärung der gesteigerten Ausscheidung bei Zufuhr sehr grosser Askorbinsäuremengen denkbar. Weder im Selbstversuch mit Sulfanilamid noch bei den Tierversuchen haben indessen so erhebliche Veränderungen der Harnmengen beobachtet werden können, dass sie die gesteigerte Ausscheidung der zyklischen Verbindung zu erklären vermöchten.

In den Tierversuchen kommt ausserdem hinzu, dass alle Tiere gleich grosse Mengen Wasser bekommen und diese unabhängig von der Askorbinsäurezufuhr vollständig konsumiert haben.

Vergleicht man die in vivo gewonnenen Ergebnisse mit denen der Versuche in vitro, so stellen wir eine weitgehende Übereinstimmung fest.

In beiden Fällen erfolgt die Umwandlung unter Bildung von Urochrom A, in beiden Fällen war auch eine Hemmung der Umwandlung bei überschüssiger Askorbinsäure zu beobachten.

Weder in vivo (bei Meerschweinchen) noch in vitro konnte bei Zufuhr von Sulfanilamid ein gesteigerter Verbrauch von Vitamin C festgestellt werden. (In vivo konnte dasselbe für Natriumsalizylat und Phenol beobachtet werden. Die zusammen mit Forssman und Frykholm untersuchten benzolexponierten Arbeiter hatten dagegen einen erhöhten Bedarf an Vitamin C. Möglicherweise spielen hier die in Kap. VI erörterten Verhältnisse eine Rolle.)

In vitro konnte keine Koppelungsreaktion zwischen zyklischer Verbindung und Askorbinsäure nachgewiesen werden, und die Koppelungsreaktionen in vivo vollzogen sich unabhängig von der C-Vitaminzufuhr. In vitro zeigte es sich, dass die Desaminierung von Histidin durch einen Phenolzusatz gehemmt werden konnte. Dasselbe wurde in vivo festgestellt: bei Zufuhr von Phenol stieg die Histidinausscheidung im Harn von Ratten.

Der Nachweis, dass die Histidinumwandlung in vivo durch Zufuhr einer anderen zyklischen Verbindung, Phenol, gehemmt wird, dürfte möglicherweise für das Verständnis der pharmakologischen Wirkung

gewisser in dieser Arbeit berührter Verbindungen von Interesse sein. Zwar kennt man spezifische Enzymsysteme für verschiedene zyklische Verbindungen. Als Beispiel ist die vor allem in der Leber vorkommende Histidase zu nennen, die Histidin abbaut. Die Hemmung des Histidinabbaus und die sich daraus ergebende Steigerung der Histidinausscheidung nach Zufuhr von Phenol kann also auch in einer Hemmung der Histidasenwirkung bestanden haben. In früheren Untersuchungen (Ekman 1941) konnte die Histidinausscheidung jedoch auch durch blosse Veränderungen der C-Vitaminzufuhr beeinflusst werden, und es erscheint möglich, dass es neben spezifischeren Enzymsystemen auch ein allgemeineres Oxydationssystem für zyklische Verbindungen gibt und dass Askorbinsäure zu diesem System gehört. Die Kapazität des Systems würde dann durch Schwankungen der Askorbinsäurezufuhr beeinflusst werden: eine vermehrte Askorbinsäurezufuhr hat eine vermehrte Umwandlung zyklischer Verbindungen zur Folge.

In der Schrifttumsübersicht wurden Untersuchungen erwähnt (S. 35), aus denen hervorging, dass Atophan durch Askorbinsäure entgiftet werden kann. In bezug auf die pharmakologische Wirkung des Atophans weiss man, dass nach Einverleibung von Atophan die Harnsäureausscheidung im Harn zunimmt. Der diesem Geschehen zugrunde liegende Mechanismus ist unbekannt. Doch erscheint es denkbar, dass analog dem Versuch mit Phenol-Histidin das Atophan den Harnsäurestoffwechsel im Organismus hemmt, was eine vermehrte Ausscheidung im Harn zur Folge hat.

Betreffs des Sulfanilamids und ähnlicher Präparate hat man vermutet, ihre Wirkung beruhe darauf, dass die Bakterien, die gegen Sulfanilamid empfindlich sind, zu ihrem Wachstum Paraaminobenzoesäure benötigen. Man nimmt an, dass Paraaminobenzoesäure als Bestandteil eines Enzymsystems der Bakterien vorhanden ist, aus diesem System aber durch das chemisch nahverwandte Sulfanilamid verdrängt werden kann. Das Sulfanilamid vermag indessen nicht die Funktion der Paraaminobenzoesäure zu übernehmen. (S. z. B. Domagk, 1942.)

Es erscheint möglich, diese Theorie mit der hier geführten Überlegung zu kombinieren. Man kann annehmen, dass der Umsatz von Paraaminobenzoesäure für die Bakterien notwendig ist. Ebenso aber wie beim Phenol/Histidin wird dieser Umsatz durch Zufuhr von Sulfanilamid verhindert.

Falls die obigen Überlegungen stichhaltig sind, muss sich die Paraaminobenzoesäure durch Askorbinsäure umwandeln lassen, und diese Umwandlung würde durch die Zufuhr von Sulfanilamid gehemmt werden. Paraaminobenzoesäure kann man indessen mit demselben Verfahren wie Sulfanilamid bestimmen, weshalb ein Konkurrenzversuch nicht möglich war. Statt dessen ist die Umwandlung von Paraaminobenzoesäure und Sulfanilamid unter identischen Verhältnissen und mit Zusatz derselben Askorbinsäuremenge untersucht worden. Es zeigte sich (Tab. 53), dass

TABELLE 53.

Vergleich zwischen der Umwandlung von Paraaminobenzoesäure (PAB) und der von Sulfanilamid durch Askorbinsäure.

Kolbenversuche. Zusatz in sämtl. Versuchen 176 mg Askorbinsäure, 13,6 mg Ferrizitrat. pH 5,8. Versuchsdauer 17 Stdn. In sämtl. Versuchen ist das molare Konzentrationsverhältnis Askorbinsäure/aromatische Verbindung/Ferrizitrat = 2 : 1 : 0,1. In sämtl. Versuchen wurde die Askorbinsäure vollständig oxydiert.

Nr.	Zusatz in mg	Umwandlung in mg	Umwandlung in % der zugesetzten Menge	Urochrom A E
	PAB			
1—2	68,55	18,3	26,6	0,19
	Sulfanilamid			
3—4	86	48,7	56,6	0,00

Paraaminobenzoesäure von Askorbinsäure umgewandelt werden kann, dass diese Umwandlung aber nur halb so gross ist wie die des Sulfanilamids. Demnach würde wohl bei einem Konkurrenzversuch in erster Linie das Sulfanilamid umgewandelt werden und also den Umsatz der Paraaminobenzoesäure hemmen.

Bei einem Vergleich der Versuche in vitro mit denen in vivo überrascht es zu sehen, dass ein normaler Umsatz zyklischer Verbindungen auch bei sehr geringer Zufuhr von Askorbinsäure aufrechterhalten wird, während in den Versuchen in vitro wenigstens eine gleich grosse Menge Askorbinsäure wie zyklische Verbindung erforderlich ist, damit eine grössere Menge der letzteren umgewandelt wird. In vivo sind die Verhältnisse aber anders, und von speziellem Interesse ist das Vorhandensein von Sulfhydrylverbindungen, die oxydierte Askorbinsäure zu reduzieren vermögen, so dass eine kleine Menge Askorbinsäure mehrere Male für dieselbe Reaktion verwendet werden kann. Solche Regenerationssysteme für Askorbinsäure sind beispielsweise in Untersuchungen von Hopkins (1936) und Borsook et al. (1937) behandelt worden. Es verdient hervorgehoben zu werden, dass in sämtlichen Fällen, in denen mit Askorbinsäure in vivo

eine Wirkung erzielt worden ist, diese mit Konzentrationen erreicht werden konnte, die nicht über denen liegen, die als normal gelten können. In den Meerschweinchenversuchen wurden 10—20 mg/Tag gereicht, in den Selbstversuchen zwischen 100 und 200 mg/Tag genommen.

Schliesslich sei erwähnt, dass die Untersuchungen über die Ausscheidung von Indol und Indoxylschwefelsäure auch von einem anderen Gesichtspunkt als dem der Beeinflussung der Ausscheidung durch Askorbinsäurezufuhr Interesse haben.

In den Versuchen mit konstanten Tagesgaben von 20 mg Indol stieg die Ausscheidung von Indoxyl, als Indoxylschwefelsäure berechnet, durchschnittlich um 10—15 mg, so dass also 25—40 % des einverleibten Indols zu Indoxylschwefelsäure umgewandelt wurden was mit den (S. 123) angeführten Literaturangaben übereinstimmt. Da die Indolausscheidung, die gleichzeitig kontrolliert wurde, konstant nur $0{,}_1$—$0{,}_2$ mg pro Tag betrug, kann als erwiesen gelten, dass der Organismus noch eine andere Möglichkeit hat, Indol zu eliminieren, als es in Form von mit einer Säure gepaartem Indoxyl auszuscheiden.

Zusammenfassend dürfte man sagen können, dass in diesem Kapitel vorgelegte Resultate von Untersuchungen in vivo und in vitro zeigen, dass zyklische Verbindungen durch Askorbinsäure unter Bildung von Urochrom A umgewandelt werden können. Sowohl in vivo als in vitro wird diese Umwandlung durch einen Überschuss von Vitamin C gehemmt, was von Interesse bei der Diskussion der optimalen Vitamin-C-Zufuhr sein dürfte. Die Untersuchungen in vitro wurden auf S. 117—121 vor dem Hintergrund ähnlicher Untersuchungen anderer Autoren besprochen, mit Versuchen über Entgiftung verschiedener Toxine durch Vitamin C und mit Angaben über einen Antagonismus zwischen Vitamin C und gewissen Hormonen verglichen.

Die Untersuchungen in vivo lassen sich mit den Arbeiten von Sealock et al. (siehe S. 26) über den Stoffwechsel des Phenylalanins und Thyrosins sowie ihrer Abkömmlinge vergleichen, ferner mit der Arbeit Beyers (siehe S. 33) über den Benzedrinstoffwechsel bei wechselnder C-Vitaminzufuhr. Eine weitere

Bestätigung finden sie durch die in Kap. I gemachten Schrifttumsangaben über Entgiftung mehrerer zyklischer Verbindungen durch Vitamin C sowie die Beeinflussung des C-Vitaminstoffwechsels durch gewisse zyklische Verbindungen. Ferner sei daran erinnert, dass z. B. MEYER (1937) auf die Ähnlichkeit zwischen Skorbut und Benzolvergiftung aufmerksam gemacht hat. LEIBOWITZ und GUGGENHEIM (1938) nahmen an, der Skorbut sei eine Vergiftung durch Stoffe, die normalerweise von der Askorbinsäure entgiftet würden. Von grundsätzlicher Bedeutung scheint die Beobachtung SEALOCKS (s. S. 27) zu sein dass das Vermögen der d-Askorbinsäure, den Tyroxinstoffwechsel zu beeinflussen, in direktem Verhältnis zur antiskorbutischen Wirkung derselben Askorbinsäureform stand.

Die Annahme scheint damit berechtigt, dass die Umwandlung zyklischer Verbindungen eine wesentliche Aufgabe des Vitamins C bildet.

KAP. IV.

Die Umwandlung zyklischer Verbindungen durch Askorbinsäure ist ein oxydativer Vorgang, auf den, ohne Mitwirkung der Askorbinsäure, eine Urochrom-A-Bildung folgt.

I. Die Umwandlung.

Im vorigen Abschnitt wurde gezeigt, dass zyklische Verbindungen durch Askorbinsäure umgewandelt werden können, doch wurde die Art der Umwandlung noch nicht näher untersucht.

Um den chemischen Ablauf im einzelnen klarzustellen, erschien es am einfachsten, nach Möglichkeit die Umwandlungsprodukte der Versuche in vitro zu isolieren. Ein solches Umwandlungsprodukt ist bereits nachgewiesen worden, nämlich das Urochrom A des Harns, ohne dass damit jedoch der Reaktionsmechanismus erklärt wäre.

Eine Schwierigkeit der Versuche, die Umwandlungsprodukte zu isolieren, lag darin, dass auch nach beendigtem Umwandlungsversuch noch ein Teil der zyklischen Verbindung übrig war, und bei Extraktionsversuchen und anderen Isolierungsverfahren zeigte es sich, dass es sehr schwierig war, diesen Rückstand von den unbekannten Umwandlungsprodukten zu scheiden.

In einer früheren Arbeit (Ekman 1942) konnte gezeigt werden, dass wahrscheinlich auch Benzol eine Umwandlung erfährt: bei einem in gewohnter Weise ausgeführten Versuche in vitro, doch mit einer Benzolaufschwemmung als zyklischer Verbindung, trat wie bei Versuchen mit anderen zyklischen Verbindungen auch hier Urochrom A auf.

Gerade ein Benzolversuch erschien sehr geeignet, die Verhältnisse näher zu studieren: durch die Flüchtigkeit des Benzols und seine relative Unlöslichkeit in Wasser wird man in der Versuchslösung bloss teils das Abbauprodukt der Askorbinsäure nebst Puffer und Katalysator, teils die Umwandlungsprodukte des Benzols erhalten. Diese letzteren dürften wesentlich andere Eigenschaften haben als die erstgenannten Verbindungen.

Versuche.

Um die Benzolsättigung der Versuchslösungen zu gewährleisten und zugleich nach Möglichkeit die Versuchseinrichtung der früheren Versuche beizubehalten, wurde ein Zweiliterkolben folgendermassen montiert:

Ein in die Mündung des Kolbens passender Stöpsel wurde mit zwei Löchern versehen, das eine für eine Kapillare, deren eines Ende gleich oberhalb des Kolbenbodens mündete, während das andere Ende mit der Luft frei in Verbindung stand, das zweite Loch für einen Rücklaufkühler, dessen eines Ende gleich unter dem Stöpsel in den Kolben mündete, während das andere Ende mit einer Wasserstrahlpumpe in Verbindung stand. Der Kolben wurde dann etwa zu drei Vierteln mit den Versuchslösungen, die mit dest. Wasser verdünnt waren, beschickt und dann mit Benzol gefüllt. Schaltete man die Wasserstrahlpumpe ein, so entstand eine Durchströmung von Luft, die teils die Sauerstoffsättigung der Versuchslösungen sicherstellte, teils aber auch durch das starke »Umrühren» die Lösungen mit Benzol sättigte. Verdunstendes und mit dem Luftstrom entweichendes Benzol wurde im Kühler kondensiert und floss in den Kolben zurück. Der Kolben stand im Wasserbad bei einer Temperatur von 42°.

Mit dieser Vorrichtung wurden folgende Versuche gemacht:

1. Mit einem Zusatz von 15 g Askorbinsäure (die mit NaOH auf pH 5,8 neutralisiert wurde), 272 mg Ferrizitrat und 500 ccm Phosphatpuffer, mit pH 5,8 und einer Versuchsdauer von 48 Stunden.

2. Wie 1., doch ohne Ferrizitrat.

In beiden Fällen entstand nach abgeschlossenem Versuch eine dunkel braunrote Wasserphase und eine rote Benzolphase. Es konnte angenommen werden, dass das Benzol während des Versuchs auch als ein Extraktionsmittel aufgetreten war, weshalb die Benzolphase eingedampft wurde. Es bildete sich ein dunkel braunroter Rückstand, in welchem nach einiger Zeit nadel- und blattförmige Kristalle auftraten. Es konnte ferner angenommen werden, dass eine fortgesetzte, effektivere Extraktion eine grössere Ausbeute liefern würde. Die Wasserphase wurde daher mit Salzsäure angesäuert und in einem Flüssigkeitsextraktionsapparat mit Äther extrahiert. Auch jetzt wurde nach Eindampfen des Extraktionsmittels ein

braunroter zäher Rückstand erhalten, der nach einiger Zeit grösserenteils in Nadeln kristallisierte. (Wurde derselbe Versuch ohne Zusatz von Askorbinsäure ausgeführt, verblieb sowohl Benzol- als Wasserphase ungefärbt. Wurde in einem Kontrollversuch eine vielfach grössere Benzolmenge eingeengt, blieb praktisch kein Rückstand zurück.)

In Versuchen in vivo hat JAFFÉ (1909) gezeigt, dass man bei Kaninchen, die Benzol einverleibt bekommen, Mukonsäure im Harn nachweisen kann. Diese Versuche haben bisweilen bestätigt werden können (FUCHS und SOOS 1916 [am Menschen] sowie THIERFELDER und KLENK 1924), während z. B. NEUMAERKER 1923 den JAFFÉschen Versuch nicht wiederholen konnte. Einen endgültigen Beweis dafür, dass wirklich eine Umwandlung zu Mukonsäure stattfindet, dürften DRUMMOND und FINAR (1938) erbracht haben, die mit einer wohlkontrollierten Methodik zu demselben Ergebnis kamen wie JAFFÉ. Indessen bildeten sich nur sehr kleine Mengen; höchstens 1 % des zugeführten Benzols wurde als Mukonsäure wiedergefunden.

Seit langem ist es bekannt (siehe NEUBAUER-HUPPERT 1913), dass man bei Einverleibung von Benzol, doch z. B. auch von Phenol, eine Ausscheidung von Brenzkatechin und Hydrochinon im Harn beobachten kann. Als es nun galt, die in den obengenannten In-vitro-Versuchen erhaltenen Kristalle zu identifizieren, wurde deshalb auch in erster Linie untersucht, ob es sich bei ihnen um Mukonsäure oder ein Polyphenol handeln könne.

Es zeigte sich, dass es nicht Mukonsäure sein konnte, während dagegen die Kristalle die üblichen Polyphenolreaktionen gaben: mit Phosphormolybdensäure wurde eine blaugrüne Farbe erhalten, die bei einem Zusatz von Ammoniak tiefblau wurde, mit Eisenchlorid und Sulfanilsäure erhielt man eine dunkel braunrote Farbe.

Es wurde auf verschiedene Weise versucht, die Kristalle aus dem braunroten Sirup zu befreien, in dem sie sich bildeten. Der einzig gangbare Weg war der, die Masse eintrocknen zu lassen und dann zu sublimieren. Es wurde dann ein weisses kristallinisches Sublimat erhalten, das bei der Elementaranalyse Daten lieferte, die völlig mit denen des Brenzkatechins und Hydrochinons übereinstimmen. (Berechnet für $C_6H_6O_2$: C =

65,42 %, H = 5,49 %. Im Präparat aus dem Versuch mit Ferrizitrat: C = 65,3 %, H = 5,48 %, im Präparat aus dem Versuch ohne Ferrizitrat: C = 65,35 %, H = 5,47 %. Alles Mittelwerte aus Doppelbestimmungen.)

Die Schmelzpunktbestimmung ergab, dass es sich um ein Gemisch von Hydrochinon und Brenzkatechin handeln muss. Der Nachweis dieser Umwandlungsprodukte erhärtet, dass das Benzol oxydiert worden ist.

Benzol ist eine wenig reaktionsbereite und recht schweroxydable Verbindung. Der Nachweis, dass der Benzolkern direkt durch Askorbinsäure oxydiert werden kann, dürfte also ein gewisses theoretisches Interesse haben, doch dürfte er auch die Annahme rechtfertigen, dass auch in denjenigen Fällen, in denen ich eine Umwandlung von Benzolabkömmlingen durch Askorbinsäure habe nachweisen können, eine Oxydation durch die Askorbinsäure vorgelegen hat.

Edlbacher und v. Segesser (1937 a) konnten aus ihren Versuchen mit Histidin folgern, dass der Imidazolring eine oxydative Spaltung erfährt. Ob in dem obigen Versuch das Benzol wirklich gespalten worden ist, lässt sich noch nicht entscheiden; eine Spaltung zu Mukonsäure hat jedenfalls nicht erwiesen werden können.

Die Versuche mit Indol und Askorbinsäure (Kap. III) weisen so grosse Ähnlichkeit mit den Versuchen zur Umwandlung anderer zyklischer Verbindungen auf, dass man wohl annehmen dürfte, dass es sich auch hier um eine Oxydation handelt.

Ein denkbares Oxydationsprodukt wäre Indoxyl, das aber in vitro nicht hat festgestellt werden können, und bei den Versuchen in vivo trat bei Vitamin-C-Mangel eine gesteigerte Indoxylausscheidung ein. Trotzdem aber könnte man annehmen, dass ein oxydativer Prozess vorliegt, dass dieser aber sowohl in vivo als in vitro nicht mit der blossen Bildung des wenig beständigen Indoxyls abgeschlossen ist. Dies könnte dann erklären, wieso in vivo die Ausscheidung von Indoxylschwefelsäure bei C-Vitamin-Mangel zunimmt: zwar hat eine Oxydation zu Indoxyl stattfinden können, für die weitere Oxydation aber hat die vorhandene Askorbinsäure nicht gereicht.

II. Die Farbstoffbildung.

Wie schon erwähnt wurde, hat SZENT-GYÖRGYI (1925, 1930) gezeigt, dass die bei welkenden Pflanzen auftretende braunschwarze Färbung ausbleibt, wenn Askorbinsäure anwesend ist. Und er erklärt dies damit, dass die Färbung dadurch bedingt sei, dass Polyphenole zu Chinonen oxydiert werden, die dann durch Kondensation und eventuelle Bindung an die Eiweissstoffe farbige Verbindungen ergeben. Die Oxydation der Polyphenole wird indessen durch Askorbinsäure verhindert.

Da hier gezeigt werden konnte, dass es sich bei der Umwandlung zyklischer Verbindungen durch Askorbinsäure um eine Oxydation handelt, die, wenn von einer Benzolverbindung ausgegangen wird, gerade zur Entstehung von Polyphenolen führt, sowie dass eine solche Oxydation auch bei Pflanzen stattfinden kann (EKMAN 1942), liegt es nahe, auch die Urochrom-A-Bildung von dem SZENT-GYÖRGYIschen Gesichtspunkt aus zu betrachten.

Versuche.

Aus den Lösungen der vorhin geschilderten Askorbinsäure-Benzol-Versuche konnte Urochrom A in gewohnter Weise isoliert werden.

Um den Vergleich mit den nach SZENT-GYÖRGYIS Theorie vorliegenden Verhältnissen weiter zu verfolgen, wurde eine Chinonlösung mit Ferrizitrat versetzt und 48 Stunden lang ein Luftstrom hindurchgeleitet. Die Lösung nahm den gewohnten braunroten Ton an, und es erwies sich als möglich, Urochrom A wie aus dem Askorbinsäure-Benzol-Versuch zu isolieren.

Auf Grund dieser Versuche wurde folgende Theorie über die Bildung des Urochroms A aufgestellt:

Bei der Oxydation zyklischer Verbindungen werden Oxydationsprodukte gebildet, die, wenn die zyklische Verbindung ein Benzolabkömmling ist, Polyphenolnatur haben. Diese Oxydationsprodukte können weiteroxydiert werden und liefern dann Urochrom A, möglicherweise durch eine Kondensation. Ist indessen Askorbinsäure im Überschuss anwesend, so wird diese Weiteroxydation und damit auch die Urochrom-A-Bildung gehemmt.

TABELLE 54.

Zunahme der Polyphenolausscheidung bei Zufuhr von Benzol (Druckereiarbeiter).

Einrichtung der Versuche: Siehe S. 131 und 134.

	Vor der Vitamin-C-Belastung		Tage nach Beginn der Vitamin-C-Belastung			
			4		11	
	E Briggs	Anzahl Bestimm.	E Briggs	Anzahl Bestimm.	E Briggs	Anzahl Bestimm.
Kontrolle 22 Arbeiter	0,135	38	0,11	20	0,12	19
Benzol 21 Arbeiter	0,25	44	0,26	17	0,21	19

(Die Oxydationsprodukte des Indols und der Imidazolverbindungen sind noch unbekannt. Auch Indol enthält jedoch einen Benzolkern, und es ist also denkbar, dass auch diese Produkte Polyphenolcharakter haben. Dagegen gibt die Theorie keine Erklärung für die Entstehung ähnlicher Farbstoffe aus Imidazolverbindungen, wenn man nicht voraussetzt, dass nach der Bildung von Chinon aus den Polyphenolen eine Spaltung des Benzolkerns stattfindet, und dass man ähnliche Spaltungsprodukte aus sämtlichen zyklischen Verbindungen erhält.)

Falls die obige Theorie richtig ist, muss man in vivo folgendes finden:

1. Bei Zufuhr einer aromatischen Verbindung muss man eine gesteigerte Ausscheidung von Polyphenolen im Harn oder eine gesteigerte Ausscheidung von Urochrom A oder beides nachweisen können.

An dem auf S. 131 beschriebenen Material von dem Einfluss von Benzol ausgesetzten Arbeitern wurden Polyphenolbestimmungen gemacht. Wie aus Tab. 54 hervorgeht, schieden die betreffenden Arbeiter etwa 100 % mehr Polyphenole aus als die im gleichen Betrieb arbeitenden, aber nicht den Benzoldämpfen ausgesetzten, zur Kontrolle untersuchten übrigen Arbeiter. Tab. 51 auf S. 134 zeigt, dass gleichzeitig auch die Ausscheidung von Urochrom A um etwa 38 % gesteigert war, und im selben Abschnitt wird gezeigt, dass die Urochrom-A-Ausscheidung bei

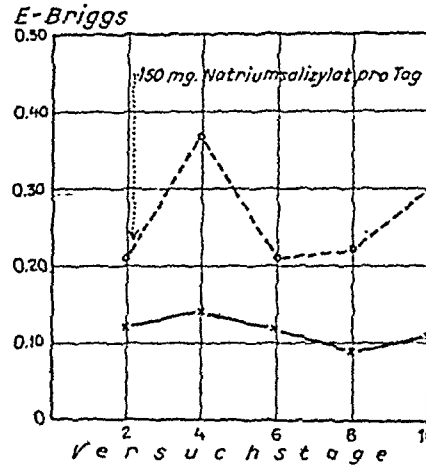

Abb. 19. *Verstärkte Ausscheidung von Polyphenolen nach Einverleibung von Natriumsalizylat.*

Der Versuch umfasst zwei Gruppen von je 4 Meerschweinchen. Natriumsalizylat per os. Tägliche C-Vitamingabe: 20 mg durch Injektion. Tagesharnmenge 25 ccm.

Versuchen in vivo auch nach Einverleibung von Indol, Salizylsäure und Sulfanilamid zunahm. Eine zunehmende Polyphenolausscheidung hat ausserdem nach Einverleibung von Phenol, Natriumsalizylat (Abb. 19) und Sulfanilamid festgestellt werden können (Abb. 19).

(Bei der Polyphenolbestimmung an den Benzolarbeitern wurde kontrolliert, dass die Harne keine Askorbinsäure enthielten; die Steigerung der Urochrom-A-Ausscheidung beläuft sich bloss auf etwa ein Drittel des Anstiegs der Polyphenolausscheidung. Die Werte der Polyphenolausscheidung können also (siehe S. 87) als zuverlässig gelten.)

2. In Skorbutversuchen kann man damit rechnen, dass das aufgespeicherte Vitamin C allmählich aufgebraucht wird. Eine Zeitlang wird also die Polyphenolbildung noch fortdauern können, wenn aber kein C-Vitaminüberschuss mehr da ist, so sind eine fortgesetzte Oxydation der Polyphenole und erhöhte Urochrom-A-Werte die Folge. Bei fortschreitendem Skorbut werden auch weiterhin die Polyphenole zur Urochrom umgewandelt, da aber die Askorbinsäure allmählich zu Ende geht, muss die Polyphenolbildung abnehmen und damit auch die Urochrom-A-

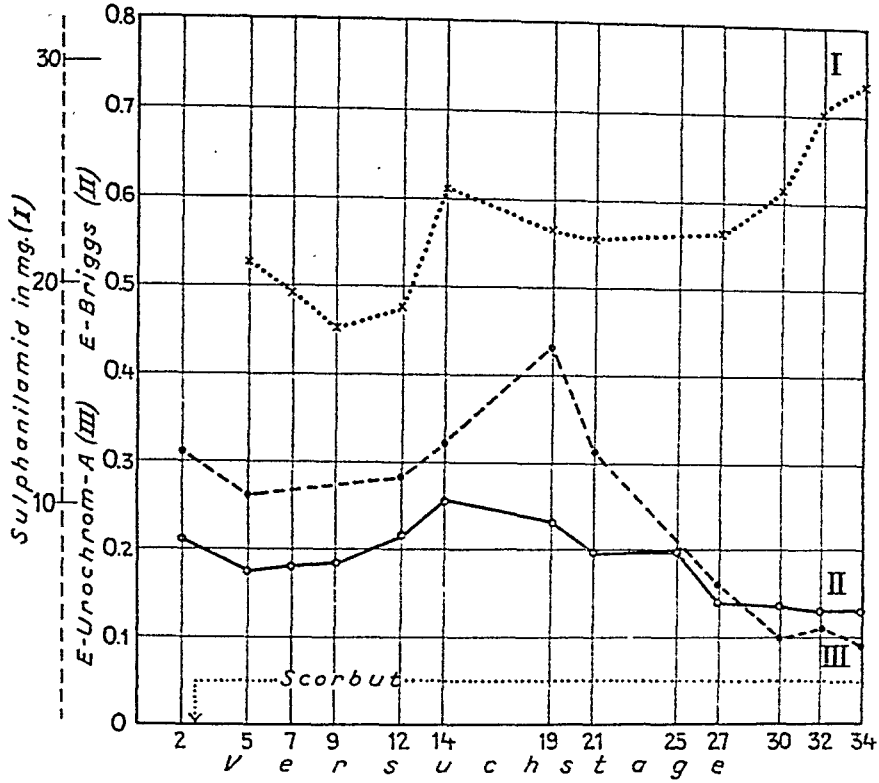

Abb. 20. *Die Ausscheidung von Sulfanilamid, Polyphenolen und Urochrom A während eines Skorbutversuchs mit Meerschweinchen.*

Die Kurven repräsentieren die Mittelwerte aus den Bestimmungen an 5 Meerschweinchen, die täglich 50 mg Sulfanilamid per os bekamen. Der Harn wurde in Zweitagesperioden gesammelt. Tagesharnmenge 25 ccm.

Bildung. Ferner wird man mit einer erhöhten Ausscheidung zyklischer Verbindungen rechnen können.

Aus Abb. 20, in der die Verhältnisse bei einer Gruppe von Meerschweinchen mit Tagesgaben von 50 mg Sulfanilamid dargestellt sind, geht hervor, dass dies auch zutrifft. Mit fortschreitendem Skorbut steigt die Sulfanilamidausscheidung, und nach einer vorübergehenden Steigerung der Urochrom-A-Ausscheidung im Anfang des Versuches nimmt die Menge des ausgeschiedenen Urochroms A und der Polyphenole ab. Da es sich hier um einen Skorbutversuch handelt, bei welchem kein Vitamin C mit dem Harn ausgeschieden wird, dürften also die Polyphenolwerte zuverlässig sein.

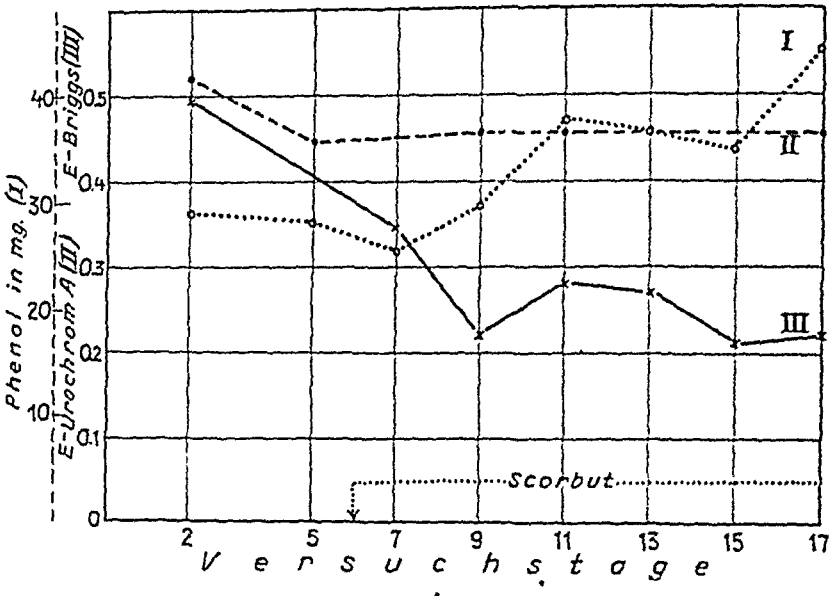

Abb. 21. *Die Ausscheidung von Phenol, Polyphenolen und Urochrom A während eines Skorbutversuchs mit Meerschweinchen.*

Die Kurven repräsentieren die Mittelwerte aus den Bestimmungen an 5 Meerschweinchen, die täglich 168 mg Phenol per os bekamen. Der Harn wurden in Zweitagesperioden gesammelt. Tagesharnmenge 25 ccm.

Ein analoges Ergebnis ist in Abb. 21 dargestellt. Die betreffenden Tiere bekamen statt des Sulfanilamids täglich 168 mg Phenol per os. Wahrscheinlich infolge der Giftwirkung des Phenols, das bei dem Askorbinsäuremangel nicht verbrannt werden kann, lebten die Tiere in diesem Versuch nur kurze Zeit, so dass eine Abnahme der Urochrom-A-Ausscheidung noch nicht in Erscheinung treten konnte.

3. Bei einer knappen, doch ausreichenden Versorgung mit Askorbinsäure wird man eine hohe Ausscheidung von Urochrom A finden können, da die Askorbinsäure zur Polyphenolbildung zwar genügt, dann aber zum Schutz der gebildeten Polyphenole nicht ausreicht. Die Polyphenolausscheidung wird also relativ gering sein.

Bei Einverleibung grosser Mengen von Vitamin C ist eine gesteigerte Polyphenolbildung zu erwarten, sowie, da der Überschuss an Askorbinsäure eine weitere Oxydation der Polyphenole

TABELLE 55.

Der Urochrom-A-Index bei wechselnder C-Vitaminzufuhr

Während des ganzen Versuchs bekamen sämtl. Tiere täglich 20 mg Phenol per os. Die Askorbinsäure wurde durch Injektion einverleibt. Während einer einwöchigen Vorperiode wurden die einzelnen Gruppen wie während des Versuchs behandelt.

Versuchstag	2	5	8	12	15
Askorbinsäure/Tag	1,5 mg				
(4 Tiere) Urochrom-A-Index	19,5	17,0	19,6	15,1	15,1
Askorbinsäure/Tag	6 mg				
(5 Tiere) Urochrom-A-Index	10,7	12,1	11,6	8,1	13,1
Askorbinsäure/Tag	24 mg		48 mg		
(4 Tiere) Urochrom-A-Index	7,0	12,5	5,6	7,4	8,1

verhindert, auch eine hohe Polyphenolausscheidung, dagegen eine geringe Urochrom-A-Ausscheidung.

In Tab. 55 ist nur die Urochrom-A-Ausscheidung verzeichnet, die sich aber der gemachten Annahme gemäss verhält: bei geringer, doch gegen Skorbut schützender Askorbinsäurezufuhr ist die Urochrom-A-Ausscheidung zunächst stark gesteigert, wird dann aber mit zunehmenden Askorbinsäuregaben immer niedriger. In Abb. 22 und 23 sind die Verhältnisse bei Zufuhr von Hydrochinon und Brenzkatechin studiert worden. Es zeigte sich, dass bei Zufuhr eines Polyphenols die Urochrom-A-Ausscheidung stark anstieg, während die Menge des ausgeschiedenen Polyphenols im Verhältnis zu den hohen Polyphenolgaben eine nur geringe Zunahme zeigte, so gering, dass man annehmen muss, dass die unter normalen Verhältnissen zu beobachtende Polyphenolausscheidung nicht durch Hydrochinon oder Brenzkatechin bedingt ist. (Berechnet man aus den E-Briggs-Werten in Abb. 23 die Polyphenolausscheidung als Hydrochionon, so

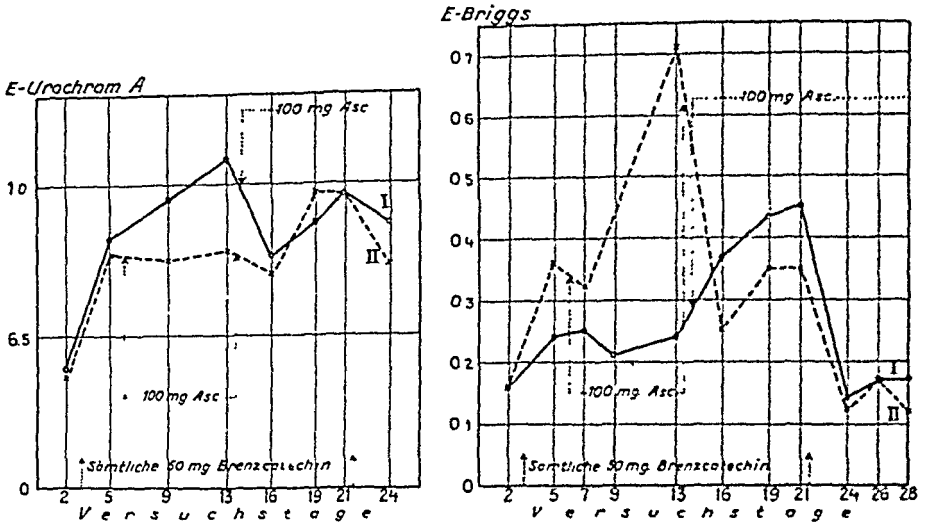

Abb. 22. *Die Ausscheidung von Urochrom A und Polyphenolen im Harn bei wechselnder Zufuhr von Brenzkatechin und Askorbinsäure.*

Der Versuch umfasst zwei Gruppen von je 6 Meerschweinchen, die während des ganzen Versuchs täglich 10 g Weisskohl bekamen (etwa 10 mg Askorbinsäure entsprechend). Weitere Askorbinsäure durch Injektion (siehe die Abb.). Brenzkatechin per os. Der Harn wurde in Zweitagesperioden gesammelt. Bei der Polyphenolbestimmung nach Briggs wurde bei der Hydrolyse kupferhaltige Salzsäure verwendet. Tagesharnmenge 25 ccm.

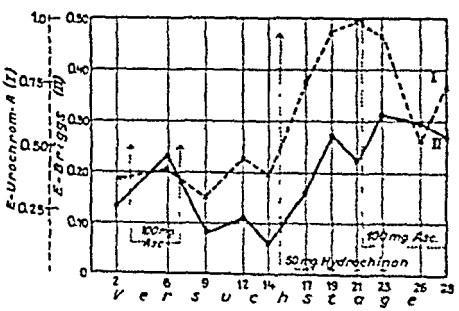

Abb. 23. *Die Ausscheidung von Urochrom A und Polyphenolen im Harn bei wechselnder Zufuhr von Hydrochinon und Askorbinsäure.*

Der Versuch umfasst eine Gruppe von 5 Meerschweinchen, die während des ganzen Versuchs täglich 10 g Weisskohl bekamen (etwa 10 mg Askorbinsäure entsprechend). Weitere Askorbinsäure durch Injektion (siehe die Abb.). Hydrochinon per os. Der Harn wurde in Zweitagesperioden gesammelt. Bei der Polyphenolbestimmung nach Briggs wurde kupferhaltige Salzsäure bei der Hydrolyse verwendet. Tagesharnmenge 25 ccm.

bekommt man bei normaler Kost eine Tagesmenge von etwa 1 mg. Nach Einverleibung von 50 mg Hydrochinon steigt die Tagesmenge auf etwa 3 mg.) Bei hohen Askorbinsäuregaben nahm die Urochrom-A-Ausscheidung ab und die Polyphenolausscheidung zu. Bei der Hydrolyse des Polyphenolharns wurde eine kupferazetathaltige Salzsäure benutzt, um die Askorbinsäure als Fehlerquelle auszuscheiden. Ein gutes Kriterium dafür, dass die gefundenen Werte durch Polyphenole und nicht durch Askorbinsäure bedingt sind, dürfte der Umstand sein, dass in Abb. 22 die Polyphenolausscheidung in beiden Gruppen an den Versuchstagen 24—28 die gleiche ist, trotzdem die eine Gruppe sehr grosse Mengen Askorbinsäure erhielt, die andere aber nicht.

Zusammenfassung der Versuchsergebnisse.

I. Bei Umwandlung von Benzol durch Askorbinsäure konnte die Bildung von Brenzkatechin und Hydrochinon nachgewiesen werden.

Es wurde daraus die Folgerung gezogen, dass der chemische Verlauf bei der Umwandlung zyklischer Verbindungen durch Askorbinsäure eine Oxydation ist.

II. In Versuchen, Benzol durch Askorbinsäure umzuwandeln, liess sich die Bildung von Urochrom A feststellen.

Wurde durch eine Chinonlösung Luft in Anwesenheit von Eisen hindurchgeleitet, so entstand Urochrom A.

Auf Grund dieser Ergebnisse wurde folgende Theorie für die Bildung von Urochrom A bei der Umwandlung zyklischer Verbindungen durch Askorbinsäure in vivo und in vitro aufgestellt:

Bei der Oxydation zyklischer Verbindungen werden Oxydationsprodukte gebildet, die, wenn die zyklische Verbindung ein Benzolabkömmling ist, den Charakter eines Polyphenols haben. Diese Oxydationsprodukte können weiter oxydiert werden und liefern dann Urochrom A, möglicherweise durch eine Kondensation. Bei Askorbinsäure im Überschuss jedoch wird diese fortgesetzte Oxydation und damit auch die Urochrom-A-Bildung gehemmt.

Folgende Versuchsergebnisse bestärken diese Annahme:

Bei Darreichung einer aromatischen Verbindung (Benzol,

Sulfanilamid, Salizylsäure, Phenol, [Polyphenole]) steigt die Ausscheidung von Polyphenolen und Urochrom A im Harn.

Bei Skorbut ist eine steigende Ausscheidung aromatischer Verbindungen und eine abnehmende Ausscheidung von Polyphenolen festzustellen. Die Urochrom-A-Ausscheidung ist anfangs erhöht und nimmt mit fortschreitendem Skorbut ab.

Bei einer ausreichenden, doch knappen Askorbinsäureversorgung findet man eine hohe Urochrom-A-Ausscheidung.

Bei Zufuhr sehr grosser Askorbinsäuremengen ist die Ausscheidung von Urochrom A vermindert, die Ausscheidung von Polyphenolen gesteigert.

Besprechung der Ergebnisse.

Bei der Besprechung der Ergebnisse aus den Versuchen in vitro in Kap. III wurde hervorgehoben, dass einige von diesen die Annahme motivieren könnten, es handle sich sowohl bei der Umwandlung der zyklischen Verbindung als bei der Farbstoffbildung um oxydative Vorgänge. Diese Annahme hat sich nunmehr bestätigt, und der chemische Verlauf ist folgender: 1) Oxydation der Askorbinsäure, 2) Oxydation der zyklischen Verbindung, 3) Farbstoffbildung durch fortgesetzte Oxydation gewisser Produkte aus 2) ohne Mitwirkung der Askorbinsäure.

Damit finden noch einige weitere Ergebnisse der Versuche in vitro ihre Erklärung.

Es konnte gezeigt werden, dass eine gewisse Übereinstimmung zwischen der Oxydation der zyklischen Verbindung und der Farbstoffbildung herrschte, jedoch keine absolute Proportionalität. Dies dürfte daran liegen, dass die Voraussetzung der Farbstoffbildung selbstverständlich die bei der Oxydation der zyklischen Verbindung gebildeten Produkte sind, dass aber die fortgesetzte Oxydation dieser Produkte keinen direkten Zusammenhang mit dem erstgenannten Vorgang hat.

Ferner hatte die Oxydation der zyklischen Verbindung ihr optimales pH zwischen $5{,}4$ und $5{,}8$, während für die Farbstoffbildung pH $6{,}6$ optimal war. Dies steht im Einklang damit, dass z. B. Polyphenole bei saurem pH weit stabiler sind als bei neutralem.

Bei sehr hohem Überschuss an Vitamin C trat eine Hemmung in der Umwandlung der zyklischen Verbindung ein. In diesen Fällen war nach beendigtem Versuch ein Rückstand von nicht oxydierter Askorbinsäure zu verzeichnen. Da die beobachteten Teilvorgänge, die Oxydation der Askorbinsäure, die Oxydation der zyklischen Verbindung und die Farbstoffbildung, wie weiter oben gezeigt worden ist, im grossen ganzen nacheinander ablaufen, kann man annehmen, dass diese Hemmung darauf beruht, dass nicht alle Askorbinsäure hat oxydiert werden können und dass daher die nach Oxydation der Askorbinsäure einsetzende Oxydation der zyklischen Verbindungen nicht zum Abschluss kommen konnte.

Die Versuchsdauer ist jedoch stets so bemessen worden, dass man annehmen darf, dass unter den obwaltenden Bedingungen mit durchströmender Luft und Gegenwart eines Katalysators alle Askorbinsäure oxydiert werden kann. YAMAMOTO (1936) indessen hat gezeigt, dass in einer Lösung, die Adrenalin (also einen Stoff von Polyphenolnatur) und Askorbinsäure enthält, nicht nur das Adrenalin, sondern auch die Askorbinsäure stabilisiert wird. Es ist möglich, dass etwas Derartiges auch bei den Versuchen in vitro stattgefunden hat, dass also bei Anwesenheit grosser Mengen Askorbinsäure nach und nach ein gewisses Gleichgewichtsverhältnis zwischen den gebildeten Oxydationsprodukten (Polyphenolen) und der Askorbinsäure eintritt, wodurch beide vor Oxydation geschützt werden.

Dasselbe ist wahrscheinlich in vivo der Fall: Auch dort konnte bei grossem Askorbinsäureüberschuss eine Hemmung in der Umwandlung der zyklischen Verbindungen festgestellt werden.

Die wesentlichen Ergebnisse dieses Kapitels dürften sich folgendermassen zusammenfassen lassen: Zyklische Verbindungen können in vivo und in vitro durch Askorbinsäure oxydiert werden. Die gebildeten Oxydationsprodukte werden bei Askorbinsäure im Überschuss stabilisiert, während sie sonst weiter oxydiert werden, was zur Bildung von Urochrom A führt.

KAP. V.

Die Oxydation zyklischer Verbindungen durch Askorbinsäure in vitro lässt sich erklären durch die Bildung von Wasserstoffsuperoxyd bei der Oxydation der Askorbinsäure selbst.

Im vorigen Kapitel konnte gezeigt werden, dass die Umwandlung zyklischer Verbindungen durch Askorbinsäure ein oxydativer Vorgang ist. In dem Schrifttumsüberblick in Kap. I wurde die Theorie von HOLTZ und TRIEM (1937 a) erwähnt, dass sich die Desaminierung von Histidin durch Askorbinsäure durch die von ihnen nachgewiesene Bildung von Wasserstoffsuperoxyd oder anderen Peroxyden bei der Oxydation der Askorbinsäure erklären lasse. HOLTZ benutzte »die starke Chemiluminiszenz, die in Lösungen von 3-aminophtalsäurehydrazid (Luminol) unter dem Einfluss von Wasserstoffsuperoxyd bei Gegenwart von Spuren katalysierenden Hämins auftritt, zum Peroxydnachweis».

In einer zur selben Zeit erschienenen Arbeit wies HUSZAK (1937) die Bildung von Wasserstoffsuperoxyd bei der Oxydation der Askorbinsäure nach, und er gibt an, dass für jedes Molekül Askorbinsäure ein Molekül Wasserstoffsuperoxyd gebildet werde. HUSZAK konstatierte die Bildung von Wasserstoffsuperoxyd dadurch, dass bei Gegenwart von Cerium(III)hydroxyd eine ziegelrote Fällung von Cerium(IV)peroxyd gebildet wurde. Er bestimmte die Wasserstoffsuperoxydbildung quantitativ, indem er die Sauerstoffentwicklung in einem WARBURGschen Respirometer nach Zusatz von Katalase mass.

In der Literatur finden sich zahlreiche Beispiele dafür, dass zyklische Verbindungen in vitro von Wasserstoffsuperoxyd oxy-

diert werden können. Nach WIELAND und MACRAE (1931) wird Harnsäure oxydiert, nach KAR (1937) Phenole und Polyphenole, nach ARNOLD, ARNOLD und LARSSON (1940) Naphtalin. RAPER (1932) zeigte, dass Thyrosin, Thyramin und Phenylalanin durch Wasserstoffsuperoxyd mit Ferrosulfat als Katalysator zu Dihydroxyphenylalanin, Dihydroxyäthylphenylalanin und Thyrosin oxdiert werden. JAMES (1940) oxydierte in gleicher Weise Sulfanilamid und Sulfapyridin unter Bildung eines roten Farbstoffes.

Von besonderem Interesse sind LEEDS (1881) Untersuchungen, in denen er zeigt, dass Benzol bei Kochen mit Wasserstoffsuperoxyd unter Bildung von Phenol und Oxalsäure oxydiert wird. Die Oxydation führte also in diesem Falle zu einer Spaltung des Benzolringes.

LEEDS Ergebnisse wurden von CROSS, BEVAN und HEIBERG (1900) bestätigt. Sie erhielten unter ähnlichen Verhältnissen, doch unter Verwendung von Ferrosulfat als Katalysator und mit grösseren Wasserstoffsuperoxydmengen eine gute Ausbeute an Phenol und Brenzkatechin sowie kleine Mengen Hydrochinon.

Diese Resultate stimmen mit der im vorigen Kapitel gemachten Beobachtung überein, dass bei der Oxydation von Benzol durch Askorbinsäure Brenzkatechin und Hydrochinon gebildet werden.

Es erschien daher naheliegend, dass auch bei der Oxydation von Benzol durch Askorbinsäure das Oxydationsmittel Wasserstoffsuperoxyd gewesen sei. Um die Richtigkeit dieser Annahme zu prüfen, wurde folgender Versuch gemacht.

Versuch.

In derselben Apparatur, die im vorigen Kapitel für den Benzol/Askorbinsäureversuch benutzt worden war, wurde eine wässrige Lösung von Wasserstoffsuperoxyd und Ferrosulfat mittels Durchleitung von Stickstoff mit Benzol gesättigt gehalten. In Abständen von einigen Stunden wurden weitere Mengen Wasserstoffsuperoxyd und Katalysator zugesetzt (jedesmal 1 ccm von MERCKS 30 % Perhydrol pro analysi und 100 mg Ferrosulfat). Sehr bald trat dieselbe Färbung der Benzol- und Wasserphase auf, die im Benzol/Askorbinsäureversuch zu beobachten gewesen war, und nach

24 Stunden wurde durch Eindampfen der Benzolschicht und Ätherextraktion der angesäuerten Wasserphase ein ähnlicher braunroter, teilweise kristallisierender Sirup erhalten. Aus diesem wurde durch Sublimierung ein Präparat gewonnen, das die üblichen Polyphenolreaktionen ergab. Durch Extraktion des Präparats mit kaltem und warmem Benzol konnten Brenzkatechin bzw. Hydrochinon isoliert und durch Schmelzpunktbestimmung identifiziert werden.

Auch in diesem Falle war somit Brenzkatechin und Hydrochinon gebildet worden. Aus der Wasserphase wurde Urochrom A isoliert.

Zusammenfassung der Versuchsergebnisse.

Mit der gleichen Versuchseinrichtung wie bei der Oxydation von Benzol durch Askorbinsäure wurde eine Oxydation von Benzol durch Wasserstoffsuperoxyd nachgewiesen. In beiden Fällen liessen sich die gleichen Zwischenprodukte, nämlich Brenzkatechin und Hydrochinon, und dasselbe Endprodukt, nämlich Urochrom A, isolieren. Da ausserdem sowohl Holtz als Huszak auf verschiedene Weise gezeigt haben, dass bei der Oxydation der Askorbinsäure Wasserstoffsuperoxyd gebildet wird, erscheint der Schluss berechtigt, dass die Oxydation zyklischer Verbindungen durch Askorbinsäure in vitro auf die Bildung von Wasserstoffsuperoxyd bei der Oxydation der Askorbinsäure selbst zurückzuführen ist.

Besprechung der Ergebnisse.

In Kap. III wurde gezeigt, dass Ferrizitrat bedeutend stärker katalysierend auf die Umwandlung aromatischer Verbindungen einwirkte, als Ferrochlorid und Kupferazetat. Wahrscheinlich liegt das daran, dass das dreiwertige Eisen die Askorbinsäureoxydation katalysieren kann, wobei es selbst zu zweiwertigem reduziert wird, und dass es dann in dieser Form die Oxydation der aromatischen Verbindung durch Wasserstoffsuperoxyd katalysieren kann. Kupfer katalysiert zwar die Oxydation der Askorbinsäure, dürfte aber dem Schrifttum nach nicht in so hohem Grade wie Eisen Oxydationen durch Wasserstoffsuperoxyd katalysieren.

Wasserstoffsuperoxyd ist ein starkes, doch wenig spezifisches

Oxydationsmittel, und dies befindet sich im Einklang mit der Fähigkeit der Askorbinsäure, Verbindungen sehr unterschiedlicher Zusammensetzung anzugreifen.

Ferner sei daran erinnert, dass ABDERHALDEN in seinen auf S. 24 referierten Versuchen fand, dass Aminosäuren auch durch Dehydroaskorbinsäure desaminiert werden können. Die Lösungen der Dehydroaskorbinsäure wurden indessen so dargestellt, dass Askorbinsäurelösungen einer leichten Oxydation ausgesetzt wurden. Die von ABDERHALDEN beobachtete Desaminierung dürfte also nicht auf den Einfluss der Dehydroaskorbinsäure zurückgehen, sondern eher auf zurückgebliebenes Wasserstoffsuperoxyd, das bei der Oxydation der Askorbinsäure gebildet worden war. Ähnlich scheint es in meinen auf S. 105 referierten Untersuchungen über die Umwandlung aromatischer Verbindungen bei wechselnder Versuchsdauer gewesen zu sein: auch nachdem alle Askorbinsäure irreversibel zerstört ist, dauert die Umwandlung der aromatischen Verbindungen an. Die Umwandlung muss hier also entweder durch zurückgebliebenes Wasserstoffsuperoxyd oder durch eine andere Oxydationsstufe der Askorbinsäure als Dehydroaskorbinsäure bewirkt worden sein.

Es scheint, als liessen sich die Ergebnisse in vitro durch die Annahme erklären, dass die Umwandlung eine Oxydation durch gebildetes Wasserstoffsuperoxyd sei, doch hat sich kein Beweis dafür erbringen lassen, dass der Verlauf in vivo derselbe wäre. Sowohl in vitro als in vivo ist indessen gezeigt worden, dass aromatische Verbindungen durch Askorbinsäure oxydiert werden. In beiden Fällen konnte die Bildung von Polyphenolen und Urochrom A nachgewiesen werden, und zwar unter gleichartigen Verhältnissen. Es herrscht also weitgehende Übereinstimmung zwischen den Verhältnissen in vitro und in vivo.

Eine Diskussion der prinzipiellen Frage, ob das Wasserstoffsuperoxyd im biologischen Oxydationsverlauf eine Rolle spiele, liegt ausserhalb des Rahmens dieser Arbeit. Nur einige Untersuchungen, welche die Oxydation zyklischer Verbindungen durch Askorbinsäure betreffen, seien im folgenden referiert.

In seinen schon erwähnten Untersuchungen konnte HUSZAK (1937) die Rolle der Askorbinsäure im Oxydationssystem der sog. Peroxydase-Pflanzen nachweisen, und er fasst seine Arbeit

wie folgt zusammen: »Im Safte der Peroxydase-Pflanzen reagiert der Sauerstoff mit der Askorbinsäureoxydase, er oxydiert ein Molekül von Askorbinsäure unter gleichzeitiger Bildung von einem Molekül Peroxyd reversibel. Das Peroxyd oxydiert nun unter Vermittlung der Peroxydase einen Benzopyranfarbstoff, welch letzterer wieder ein weiteres Molekül Askorbinsäure reversibel oxydiert. Hierdurch werden nun alle vier Valenzen des Sauerstoffmoleküls zur Oxydation der Askorbinsäure nutzbar gemacht, welch letztere nun wieder durch die Dehydrasen bzw. ihren Wasserstoff, event. unter Vermittlung von SH-Gruppen reduziert werden kann.»

In eigenen Untersuchungen (Ekman 1942) ist indessen gezeigt worden, dass Phenol bei Pflanzen (Kresse) durch Askorbinsäure entgiftet wird, und es erscheint möglich, dass diese Entgiftung dadurch eingetreten ist, dass statt eines Benzopyranfarbstoffs das Phenol von dem gebildeten Wasserstoffsuperoxyd oxydiert worden ist. Hierdurch würde zwar die durch den oxydierten Benzopyranfarbstoff bedingte Oxydation anderer Askorbinsäuremoleküle fortfallen. Allem Anschein nach gibt es aber ausreichende Möglichkeiten für die Askorbinsäureoxydation im System Sauerstoff-Askorbinsäureoxydase.

Eine andere denkbare Möglichkeit ist, dass bei Gegenwart von Benzopyranfarbstoffen diese gemäss der Auffassung Huszaks oxydiert werden, dass sie aber später nicht nur Askorbinsäure oxydieren können, sondern beispielsweise auch zyklische Verbindungen. Die Benzopyranfarbstoffe könnten also als »nichtenzymatische Zwischenkatalysatoren» sowohl für die Askorbinsäure selbst als auch für andere Stoffe auftreten.

Gegen alle Versuche, dem Wasserstoffsuperoxyd eine Rolle im lebenden Organismus zuzusprechen, ist der Einwand erhoben worden, das Vorhandensein von Katalase müsse eine Ausnützung eventuell gebildeten Wasserstoffsuperoxyds unmöglich machen. Huszak konnte indessen in seiner Arbeit zeigen, dass die Oxydation eines Benzopyranfarbstoffs durch Wasserstoffsuperoxyd in Gegenwart der normal in Pflanzen vorkommenden Peroxydase- und Katalasemengen stattfinden konnte. Ob entsprechendes in bezug auf die hier untersuchten zyklischen Verbindungen gilt, müssen weitere Untersuchungen ausweisen.

Die Entstehung von Wasserstoffsuperoxyd bei der Oxydation von Askorbinsäure ist auch mit einer anderen Gruppe von Untersuchungen in Zusammenhang gebracht worden.

BINGOLD (1934) konnte zeigen, dass bei Einwirkung von Wasserstoffsuperoxyd in alkalischer Lösung auf Hämin ein roter Farbstoff, das Pentdyopent, entsteht, und ferner, dass auch Hämoglobin in gleicher Weise angegriffen werden kann. BINGOLD fand weiter in ikterischem Harn eine Vorstufe dieses Pentdyopents und nahm an, dass der Katalasegehalt des Blutes gewöhnlich einen Schutz gegen einen solchen Abbau von Hämoglobin durch Wasserstoffsuperoxyd in vivo darstelle.

LEMBERG et al. (1938) fanden, dass Hämoglobin (oder Hämoglobinabkömmlinge) und Askorbinsäure bei Gegenwart von Sauerstoff der Luft eine gekoppelte Reaktion durchmachen, die zur Bildung von Värdohämatin führt.

EDLBACHER und v. SEGESSER (1937 b) haben gezeigt, dass bei der Digestion gewachsener Erythrozyten bei Gegenwart von Askorbinsäure in Phosphatpuffer pH = 7,2 bei 38° unter Durchleiten von Sauerstoff ein grüner Farbstoff entstand, der zwar nicht frei von Eisen erhalten werden konnte, trotzdem aber als ein Gallenfarbstoff angesehen werden durfte.

BINGOLD (1941) hebt hervor, dass bei Oxydation von Askorbinsäure nach HOLTZ und THIEM (1937 a) Wasserstoffsuperoxyd entsteht, und er vergleicht daher LEMBERGS sowie EDLBACHER und v. SEGESSERS Arbeiten über die Oxydation des Hämoglobins (und von Derivaten desselben) durch Askorbinsäure mit seinen eigenen Untersuchungen, bei denen das Oxydationsmittel statt dessen aus Wasserstoffsuperoxyd bestand. Er nimmt an, dass etwas Ähnliches in vivo geschehen könne, indem die Katalasehemmung der Wasserstoffsuperoxydoxydation unter gewissen Umständen weniger stark sei, und dass also die Askorbinsäure für die Bildung von Gallenfarbstoffen aus dem Hämoglobin von Bedeutung wäre.

In diesem Kapitel wird gezeigt, dass die Oxydation zyklischer Verbindungen durch Askorbinsäure in vitro auf die Bildung von Wasserstoffsuperoxyd bei der Oxydation der Askorbinsäure selbst zurückgeführt werden kann.

Es wird an den in früheren Untersuchungen vom Verfasser geführten Nachweis erinnert, dass Phenol auch bei Pflanzen durch Askorbinsäure entgiftet wird. Dieses Ergebnis wird mit der HUSZAKschen *Theorie über das Oxydationssystem der Peroxydase-Pflanzen zusammengestellt. Es wird die Annahme gemacht, dass das bei der Oxydation von Askorbinsäure gebildete Wasser-*

stoffsuperoxyd entweder direkt oder durch Vermittlung von Benzopyranfarbstoffen zyklische Verbindungen oxydiert (in beiden Fällen unter Mitwirkung von Peroxydase). Dieselbe Theorie liesse sich dann auch auf die hier vorgelegten Ergebnisse aus Tierversuchen anwenden.

KAP. VI.

Die Oxydation von zyklischen Verbindungen durch Askorbinsäure in vivo lässt sich durch Zufuhr von Vegetabilien aktivieren, die ein askorbinsäureoxydierendes System enthalten.

Wie im vorigen Kapitel gezeigt wurde, lässt sich die Oxydation von zyklischen Verbindungen in vitro durch die Bildung von Wasserstoffsuperoxyd bei der Oxydation der Askorbinsäure selbst erklären. Es wurde auf Huszaks Theorie von der Rolle der Askorbinsäure bei den sog. Peroxydase-Pflanzen hingewiesen und betont, dass diese mit gewissen Veränderungen auch auf die oben vorgelegten Tierversuche Anwendung finden könne.

Nach dieser Theorie wäre die Askorbinsäure ein Glied in einem komplizierten Oxydationssystem, bestehend aus einer Askorbinsäureoxydase, einer Peroxydase, einem nichtenzymatischen Zwischenkatalysator und einem Regenerationssystem für die Askorbinsäure. Kleine Mengen Askorbinsäure liessen sich auf diese Weise für die Oxydation zyklischer Verbindungen gebrauchen und, vorausgesetzt, dass die Regeneration in einer Reduktion der Dehydroaskorbinsäure durch Vermittlung verschiedener Dehydrasen bestände, auch für den allgemeineren Oxydationsvorgang. Es erscheint möglich, ein solches System in In-vitro-Versuchen aufzubauen, um die Annahme evtl. zu bestätigen, dass auch zyklische Verbindungen in diesem System oxydiert werden. Solche Versuche werden im Zusammenhang mit anderen Untersuchungen zur Ausführung kommen.

Von grösserem Werte zur Bestätigung der Annahme wäre indessen der Nachweis, dass sich in vivo der Umsatz zyklischer Verbindungen durch Zufuhr eines anderen Bestandteils dieses

Systems als Askorbinsäure beeinflussen liesse. In diesem Zusammenhang müssen die WACHHOLDERschen Untersuchungen genannt werden.

WACHHOLDER (1940) legte eine neue Theorie über die Bedeutung des Vitamins C vor. Er lehnte die oft gehörte Behauptung ab, dass man dem Organismus grosse Mengen Askorbinsäure zuführen müsse, um ein stark reduzierendes Milieu in den Geweben aufrechtzuerhalten, und betonte, dass es statt dessen »darauf ankommt, dass der Verbrauch (Umsatz) des Vitamins nicht unter ein gewisses Mass sinkt». Es gilt dabei mit der Kost nicht nur Askorbinsäure zuzuführen, sondern auch solche Stoffe, welche die Askorbinsäureoxydation fördern können.

WACHHOLDER gibt folgende Belege für seine Ansicht:

1. LAUBER und BERRSIN (1939) berichten, dass ein Skorbutpatient, der Askorbinsäure einverleibt bekam, die volle Menge wieder ausschied und keine Besserung zeigte, bis er ausserdem eine Bluttransfusion erhielt.

2. Ein Polarforscher, der von C-vitaminarmer Kost lebte, jedoch täglich 100 mg Askorbinsäure ass, bekam trotzdem typisch skorbutische Erscheinungen, die erst verschwanden, als er Obst und Gemüse bekam.

3. WACHHOLDER konnte in eigenen Versuchen zeigen, dass man bei Verabfolgung einer gewissen Tagesgabe Vitamin C als Askorbinsäure eine weit stärkere Ausscheidung im Harn erzielte, als wenn dieselbe Menge in Form von Gemüse einverleibt wurde.

Gegen diesen Versuch liesse sich jedoch einwenden, dass bei Zufuhr von Vitamin C beispielsweise in Form von askorbinsäureoxydasehaltigen Gemüsen vielleicht schon bei der Resorption Askorbinsäureverluste entstehen, die verminderte Ausscheidung im Harn erklären könnten. WACHHOLDER (1942 a) konnte indessen zeigen, dass Speichel, Magensaft und Duodenalsaft Stoffe enthielten, welche die Askorbinsäure auch bei Gegenwart von Askorbinsäureoxydase vor Oxydation schützten.

4. Ferner fand WACHHOLDER, dass von 28 Paradentosepatienten nur 60 % bei Zufuhr von Askorbinsäure eine Besserung zeigten. Es erwies sich indessen, dass die Askorbinsäureausscheidung im Harn bei denen, die geheilt wurden, bedeutend geringer war als bei den Versagern. Das heisst, dass die Versager die verabfolgte Askorbinsäure nicht haben ausnützen können, sondern sie mit dem Harn ausgeschieden haben.

Von den WACHHOLDERschen Untersuchungen ausgehend, erschien es also möglich, die Wirkungsweise der Askorbinsäure in vivo durch Zufuhr von Pflanzen, die ein askorbinsäureoxydierendes System enthalten, noch weiter zu beleuchten. Um die Fehler-

quellen zu vermeiden, die durch eine unterschiedliche Resorption synthetischer Askorbinsäure und vegetabilischen Vitamins C bedingt sein könnten, müssen indessen die Untersuchungen über den C-Vitaminumsatz an Versuchstieren ausgeführt werden, die sämtlich gleich grosse Mengen Askorbinsäure durch Injektion bekommen. An diesem Material kann man dann in Erfahrung bringen, inwieweit sich die Ausscheidung durch Zufuhr von Askorbinsäureoxydatoren beeinflussen lässt. Man kann also unter kontrollierten Verhältnissen ein Bild vom Verbrauch des Vitamins C gewinnen, und ferner, indem man gleichzeitig den Umsatz einer zyklischen Verbindung studiert, objektiv beurteilen, ob ein etwa gesteigerter C-Vitaminverbrauch auch mit gesteigerter Funktion verknüpft ist. (Eine gesteigerte Funktion muss sich dann nach meinen oben vorgelegten Ergebnissen als eine verstärkte Oxydation der zyklischen Verbindung äussern.)

Experimentelles.

Die Versuche wurden folgendermassen variiert:

1. Meerschweinchen bekamen durch Injektion täglich eine bestimmte Menge Askorbinsäure und Sulfanilamid. Ein Teil der Tiere bekam ausserdem askorbinsäureoxydasereiche Vegetabilien (Gurke und Kürbis). Wie aus Abb. 24 a und b hervorgeht, findet man bei den mit Gurke gefütterten Tieren eine verminderte Askorbinsäureausscheidung und zugleich eine verminderte Sulfanilamidausscheidung (einer gesteigerten Sulfanilamidverbrennung entsprechend). Der Unterschied tritt sowohl bei grösseren als bei kleineren Askorbinsäuregaben hervor. Reicht man statt der Gurke Kürbis, der im Verhältnis zur Gurke viermal ärmer an Oxydase ist, so steigt die Ausscheidung sowohl der Askorbinsäure als des Sulfanilamids auf den Stand der Kontrolltiere.

2. Meerschweinchen bekamen täglich eine gleichbleibende Menge Sulfanilamid. Durch Skorbutkost wurde eine erhöhte Sulfanilamidausscheidung herbeigeführt. Verschiedene Gruppen der Tiere bekamen dann gleich grosse Mengen Vitamin C, und zwar teils in Form von synthetischer Askorbinsäure, teils in Form von askorbinsäurereichen Vegetabilien, die ausserdem ein

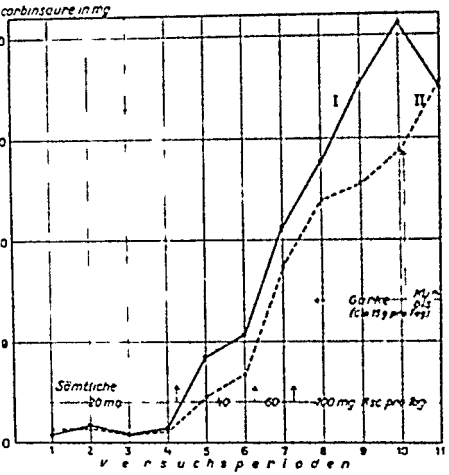

Abb. 24 a.

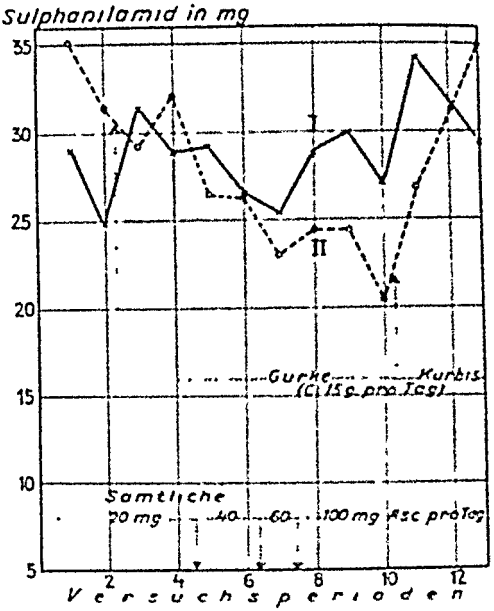

Abb. 24 b.

Abb. 24 a und b. *Die Einwirkung von askorbinsäureoxydaschaltigen Vegetabilien auf die Ausscheidung von Askorbinsäure (a) und Sulfanilamid (b) im Harn.*

Der Versuch wurde an zwei Gruppen von 4 (Gruppe I) bzw. 3 (Gruppe II) Meerschweinchen durchgeführt. Sämtliche Tiere bekamen täglich 50 mg Sulfanilamid per os. Askorbinsäure durch Injektion (siehe die Abb.). Jeden zweiten Tag wurde der Harn zur Bestimmung der Askorbinsäure gesammelt, damit abwechselnd jeden zweiten Tag zur Bestimmung von Sulfanilamid. Zwei Tage — eine Versuchsperiode. Von den dargereichten Vegetabilien hatte Schlangengurke einen 4mal höheren Oxydasegehalt als Kürbis.

askorbinsäureoxydierendes System enthalten. Wie aus Abb. 25 und 26 hervorgeht, zeigt die Sulfanilamidausscheidung bei den mit Vegetabilien gefütterten Tieren stets eine starke Abnahme, während die Tiere, die synthetische Askorbinsäure bekommen, entweder unveränderte oder nur wenig kleinere Sulfanilamidmengen ausscheiden.

Sowohl in den Versuchen nach 1. wie nach 2. wurde die Einwirkung relativ grosser Kupfer- und Eisenmengen untersucht. Keines dieser Metalle übte jedoch einen Einfluss auf die Ausscheidung von Askorbinsäure oder Sulfanilamid aus.

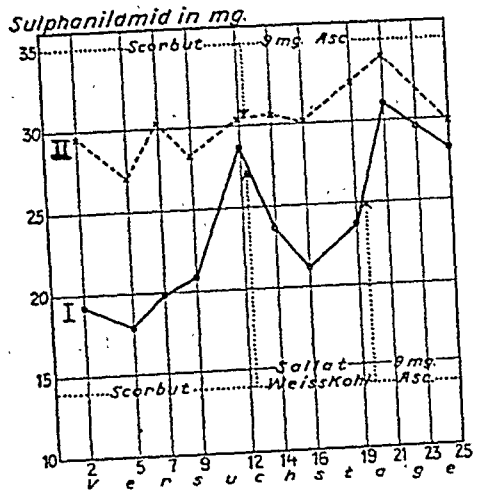

Abb. 25.

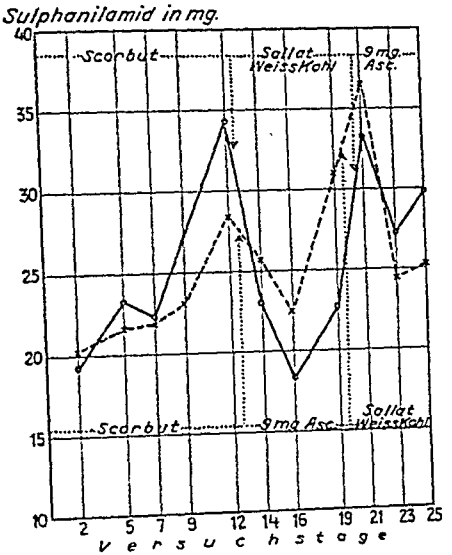

Abb. 26.

Abb. 25. *Vergleich zwischen der Einwirkung synthetischer Askorbinsäure und askorbinsäure- und askorbinsäureoxydasehaltiger Vegetabilien auf die Sulfanilamidausscheidung.*

Der Versuch wurde mit zwei Gruppen Meerschweinchen durchgeführt, Gruppe I mit 3 und Gruppe II mit 4 Tieren. Sämtliche Tiere bekamen täglich 50 mg Sulfanilamid per os. Salat und Weisskohl wurden in einer Menge gereicht, dass ihr totaler C-Vitamingehalt 9 mg Askorbinsäure entsprach.

Abb. 26. *Wie Abb. 25. — Zwei Versuchsgruppen mit je 4 Tieren.*

Zusammenfassung der Versuchsergebnisse.

Bei Meerschweinchen, die konstante Mengen Askorbinsäure und Sulfanilamid bekamen, konnte durch Zufuhr von Vegetabilien, die Askorbinsäureoxydatoren enthalten, die Ausscheidung sowohl der Askorbinsäure als des Sulfanilamids gesenkt werden. Dies dürfte bedeuten können, dass ein verstärkter Verbrauch von Vitamin C stattgefunden hat, begleitet von einer vermehrten Sulfanilamidoxydation.

Besprechung.

Wachholder (1942 b) fasst die Möglichkeiten für eine Oxydation der Askorbinsäure folgendermassen zusammen:

(1. Durch Autoxydation. Diese Möglichkeit erscheint jedoch unter physiologischen Bedingungen wenig glaubhaft.)

2. Katalyse durch Schwermetalle (Eisen und Kupfer).
3. Durch eine spezifische Askorbinsäureoxydase.
4. Eine indirekte Oxydation durch Phenole bzw. Flavone (Benzopyranfarbstoffe), nachdem diese zuvor durch Polyphenolase bzw. durch Peroxydase und Peroxyde oxydiert worden waren.
5. Durch das System Cytochrom und Cytochrom-(Indophenol-) Oxydase oder durch Hämoglobinabkömmlinge.

In den obigen Versuchen liess sich der Verbrauch von Vitamin C durch Darreichung von Vegetabilien (Gurke, Salat oder Weisskohl) beeinflussen, während die Zufuhr von Eisen und Kupfer ohne Einwirkung blieb.

Die allgemeine Ansicht dürfte sein, dass Askorbinsäureoxydase eine Eiweisskomponente enthält, weshalb es undenkbar erscheint, dass ein solches Enzym in unveränderter Gestalt im Organismus resorbiert werden und wirksam sein sollte. Wenn es sich darum handelt, die oben aufgezählten Möglichkeiten für eine Oxydation der Askorbinsäure mit irgendeinem in den verabfolgten Vegetabilien enthaltenen Stoff in Verbindung zu bringen, so erscheint als einzige Möglichkeit die Annahme, dass sie ein Phenol oder einen Benzopyranfarbstoff enthalten haben, der indirekt eine Oxydation hat herbeiführen können.

In der Einleitung dieses Kapitels wurde die Absicht ausgesprochen, Belege dafür zu finden, dass Huszaks Theorie für den Askorbinsäureumsatz der Peroxydasepflanzen auch auf den Säugetierorganismus anwendbar sei.

Huszak konnte zeigen (siehe S. 160), dass die Oxydation von Askorbinsäure in einem Peroxydasesystem ähnlich dem der sog. Peroxydasepflanzen in sehr hohem Grade durch Zufuhr von Benzopyranfarbstoffen gesteigert werden konnte. Dieselbe Steigerung, obwohl in geringerem Grade, konnte indessen auch mit einfacheren Phenolen, z. B. Brenzkatechin und Hydrochinon, erzielt werden. In den hier vorgelegten Versuchen ist es gelungen, die Oxydation von Askorbinsäure bei Meerschweinchen durch Darreichung von Vegetabilien zu beeinflussen, die solche Verbindungen enthalten, die nach der Huszakschen Theorie wirksam sind. Bei der Besprechung dieser Theorie wurde indessen auf die Möglichkeit hingewiesen, dass die von Huszak als nicht-

enzymatische Zwischenkatalysatoren aufgefassten Benzopyranfarbstoffe sowohl in die Oxydation der Askorbinsäure als z. B. in diejenige zyklischer Verbindungen eingreifen könnten.

Gewisse Beobachtungen im Laufe der experimentellen Arbeit scheinen in diesem Sinne gedeutet werden zu können.

In dem Versuch (S. 166), in dem die Ausscheidung von Askorbinsäure bei Meerschweinchen mit und ohne Zufuhr von Askorbinsäureoxydatoren verglichen wurde, bekamen die Tiere im Verlauf des Versuchs wechselnde Mengen Askorbinsäure. Es zeigte sich da, dass der C-Vitaminumsatz auch durch blosse Zufuhr grosser Askorbinsäuremengen gesteigert werden konnte. (Reichte man nämlich 20 mg täglich, so wurden im Harn 1—2 mg ausgeschieden, bei Tagesgaben von 100 mg wurden 25—40 mg ausgeschieden. In dem ersteren Falle haben die Tiere also etwa 18 mg täglich verbraucht, im letzteren Falle 60—75 mg.) Dieser gesteigerte Vitamin-C-Verbrauch hatte indessen keine entsprechend gesteigerte Verbrennung der zyklischen Verbindung im Gefolge, wenn nicht die Tiere ausserdem askorbinsäureoxydasehaltiges Material und damit auch Zwischenkatalysatoren bekamen. In diesem Zusammenhang sei an den Befund in Kap. III erinnert, dass nämlich die Zufuhr grosser Askorbinsäuremengen entweder keinen Einfluss auf den Umsatz der untersuchten zyklischen Verbindung hat oder dass dieser Umsatz bei extrem hoher Zufuhr von Askorbinsäure eine Hemmung zeigte.

Zusammenfassend dürfte man also sagen können, dass ein gesteigerter C-Vitaminumsatz an und für sich keine Steigerung einer bestimmten Funktion des Vitamins C, nämlich der Oxydation zyklischer Verbindungen, im Gefolge zu haben braucht. Eine Steigerung sowohl des C-Vitaminumsatzes als der Oxydation der untersuchten zyklischen Verbindung konnte indessen durch Zufuhr von askorbinsäureoxydasehaltigem Material bewirkt werden. Es konnte wahrscheinlich gemacht werden, dass diese Beeinflussung des C-Vitaminumsatzes auf dem Vorhandensein von Benzopyranfarbstoffen oder anderen Stoffen, die nach Huszak als nichtenzymatische Zwischenkatalysatoren auftreten können, zurückzuführen ist. Es erscheint möglich, dass diese Katalysatorwirkung nicht nur die Oxydation der Askor-

binsäure, sondern auch die Oxydation der zyklischen Verbindung betrifft.

Diese Anschauung liesse sich auch mit einigen anderen C-Vitaminproblemen in Zusammenhang bringen.

Seit SZENT-GYÖRGYI (1932) gezeigt hat, dass das Vitamin C mit der Askorbinsäure identisch ist, sind in der Literatur mehrfach Angaben erschienen, die dahin gingen, die synthetische Askorbinsäure sei nicht ebenso wirksam wie das »natürliche» Vitamin. Ein solcher Unterschied besteht selbstverständlich nicht, doch sind mehrere Untersuchungen veröffentlicht worden, die darauf hindeuten, dass in pflanzlichem Material neben der Askorbinsäure noch ein anderer Faktor vorhanden sei, der die Wirkung des Vitamins C aktivieren könnte. Solche »Hilfsvitamine» sind v. EULERS (1934) Faktor I und SZENT-GYÖRGYIS et al. (1936) Citrin. Citrin enthält gerade Benzopyranfarbstoffe, und es ist wohl möglich, dass auch der Faktor I zu den oben besprochenen Zwischenkatalysatoren gehört.

Die Schwierigkeit war bisher nur die, dass man keinen objektiven Beweis für die Existenz dieser Hilfsvitamine hat erbringen können. Es scheint, als ob die oben vorgelegten Untersuchungen, in denen es gelungen ist, den Umsatz einer zyklischen Verbindung durch einen Faktor zu beeinflussen, der in den Askorbinsäureumsatz eingreift, einen solchen Beweis darstellten. Es sind Untersuchungen im Gange, um unter Verwertung der »Oxydation zyklischer Verbindungen durch Askorbinsäure» als Test den Charakter der im Zusammenhang mit der Askorbinsäureoxydase vorkommenden nichtenzymatischen Zwischenkatalysatoren als Hilfsvitamine der Askorbinsäure näher zu untersuchen.

Bei Untersuchungen über verschiedene Probleme, die mit dem Vitamin C zusammenhängen, haben sog. Belastungsversuche grosses Interesse erregt. Man hat eine gewisse Tagesgabe Askorbinsäure einverleibt und dann untersucht, wie lange diese Gaben verabfolgt werden mussten, bis ein gewisser Prozentsatz der einverleibten Menge im Harn ausgeschieden wurde. Soweit man auf diese Weise ein Bild von der C-Vitaminsättigung des Individuums gewinnen wollte, scheint man hier eine wesentliche Fehlerquelle ganz vernachlässigt zu haben, nämlich den

u. a. hier aufgezeigten Einfluss von in der Kost enthaltenen Stoffen, welche die C-Vitaminoxydation beeinflussen können.

(Bei den weiter oben referierten Belastungsversuchen von FORSSMAN und FRYKHOLM (S. 131) liegen die Dinge anders. Dort sollte gerade ein gesteigerter C-Vitaminverbrauch nach Einverleibung von Benzol nachgewiesen werden. Der gesteigerte C-Vitaminverbrauch äusserte sich in einer Verlängerung der Zeitspanne bis zum Eintreten der »Sättigung», und er könnte durch HUSZAKS Befund erklärt werden, dass Brenzkatechin und Hydrochinon — die Oxydationsprodukte des Benzols — die Oxydation von Askorbinsäure in einem Peroxydasesystem steigern können. Die eigenen Versuche, einen erhöhten C-Vitaminverbrauch nach Zufuhr von Sulfanilamid und Natriumsalizylat nachzuweisen, missglückten (S. 130). Dies liesse sich dadurch erklären, dass die Oxydationsprodukte dieser Verbindungen nicht wie Brenzkatechin und Hydrochinon die Askorbinsäureoxydation steigern können.)

Eine Zusammenfassung der Ergebnisse dieses Kapitels zeigt, dass ein gesteigerter Umsatz und eine gesteigerte Funktion der Askorbinsäure bei Zufuhr von Vegetabilien, die Askorbinsäureoxydase und damit wahrscheinlich auch Benzopyranfarbstoffe oder andere Phenole enthalten, die als »nichtenzymatische Zwischenkatalysatoren» auftreten können, nachzuweisen war. Dies scheint vorauszusetzen, dass die Wirkung der Askorbinsäure darauf beruht, dass bei der Oxydation der Askorbinsäure selbst Wasserstoffsuperoxyd ensteht, das dann in ein Peroxydasesystem eingeht.

Es wird darauf hingewiesen, dass diese Versuche einen Beweis für die Existenz von »Hilfsvitaminen» der Askorbinsäure darstellen.

Der Wert von sog. Belastungsversuchen wird in Frage gestellt.

KAP. VII.

Vergleichende Untersuchungen über die bisher in dieser Arbeit erwähnten Urochrom-A-Präparate.

Die Urochrom-A-Ausscheidung ist in dieser Arbeit an Meerschweinchen untersucht worden, die eine normale Kost oder zusätzlich zu dieser Indol, Natriumsalizylat, Sulfanilamid, Hydrochinon oder Brenzkatechin erhielten. Am Menschen ist sie bei Zufuhr von Benzol untersucht worden. Bei Versuchen in vitro wurde gezeigt, dass Urochrom A bei der Oxydation von Indol, Salizylsäure, Sulfanilamid und Benzol durch Askorbinsäure entsteht, ferner bei der Oxydation von Benzol durch Wasserstoffsuperoxyd, sowie wenn eine Chinonlösung durch Zufuhr von Sauerstoff oxydiert wird.

In diesen Versuchen wurde unter »Urochrom A» ein Farbstoff verstanden, der sich mit der in Kap. II angegebenen Urochrom-A-Bestimmungsmethodik bestimmen und durch ein Verfahren isolieren liess (siehe unten), das sich an diese quantitative Methodik anschliesst. Diese verschiedenen Urochrom-A-Präparate werden hier zum Gegenstand einer vergleichenden Untersuchung in bezug auf Absorptionsspektren, Veränderungen der Absorptionsspektren bei Zusatz von Ammoniak und Salzsäure, die Fähigkeit, durch Schwefelwasserstoff reduziert zu werden, sowie die Fähigkeit, Dichlorphenolindophenol und Phosphormolybdensäure zu reduzieren, gemacht.

Die einzelnen Präparate werden wie folgt bezeichnet:

UA-Mensch, UA-Meerschw., womit gemeint ist, dass sie aus Menschen- oder Meerschweinchenharn bei normaler Ernährung gewonnen sind.

UA-Indol-Meerschw., UA-Sulph-Meerschw., UA-Nasal-Meer-

schw., UA-Polyphen-Meerschw., womit gemeint ist, dass sie aus dem Harn von Meerschweinchen gewonnen sind, die eine normale Kost und ausserdem Indol bzw. Sulfanilamid, Natriumsalizylat oder Polyphenole (Brenzkatechin oder Hydrochinon) bekommen haben.

UA-Indol-Asc., UA-Sulph-Asc., UA-Nasal-Asc., UA-Benzol-Asc., womit gemeint ist, dass sie aus den Versuchslösungen nach Oxydation von Indol, Sulfanilamid, Natriumsalizylat oder Benzol mit Askorbinsäure isoliert worden sind.

UA-Benzol-H_2O_2, aus der Versuchslösung nach Oxydation von Benzol mit Wasserstoffsuperoxyd isoliert.

UA-Chinon, aus der Versuchslösung nach Oxydation einer Chinonlösung mit Luftsauerstoff isoliert.

Experimentelles.

1. Isolierung von Urochrom A.

Das Urochrom A wurde nach einem Verfahren isoliert, das sich eng an die quantitative Bestimmungsmethode anlehnt und auf der auch dort benutzten Fällung mit Kupferazetat fusst:

1000 ccm Urin oder Lösung aus den Versuchen in vitro wurde mit 100 ccm 5,3 % Bariumazetat und 50 ccm 10 % Ammoniak versetzt. Anschliessend Erwärmen auf 45—50° und Abfiltern der schnell vollständigen Fällung von Sulfaten und Phosphaten. Das Filtrat wird mit der gleichen Menge 5 % Kupferazetatlösung versetzt. Nach mindestens 24 Stunden wird die Fällung, die aus der Kupferverbindung des Urochroms A besteht, gefiltert und sorgfältig mit Aq. dest. gewaschen. Die Fällung wird mit Schwefelwasserstoff gespalten und man erhält Urochrom A in wässriger Lösung. Diese wird bei 45—50° auf Sirupdicke eingedickt. Der Sirup wird (unvollständig) in 96 % Alkohol gelöst, und aus dieser Alkohollösung wird das Urochrom A mit der zwanzigfachen Menge Äther ausgefällt.

2. Bereitung der Versuchslösungen.

Mit sämtlichen Präparaten wurde folgendermassen verfahren:

Kleinere Mengen (nicht über 500 mg) der Ätherfällung wurden in 2 ccm Wasser verrührt, worauf 18 ccm abs. Alkohol zugesetzt wurden. Nach Filterung wurde mit dem zwanzigfachen Volumen Äther gefällt. Die jetzt erhaltene Fällung wurde wieder in 2 ccm Wasser verrührt, worauf abermals 18 ccm abs. Alkohol zugesetzt wurden.

Diese Urochrom-A-Lösung wurde nach vorherigem Filtern mit 90 % Alkohol verdünnt, bis im Zeiss-Pulphrichphotometer mit dem Filter S50 bei einer Schichtdicke von 10 mm und mit 90 % Alkohol als Vergleichslösung der Extinktionswert 0,32 erhalten wurde.

Vorausgesetzt, dass die Präparate, aus denen die obige Lösung dargestellt wurde, aus demselben Farbstoff bestehen, enthalten diese Lösungen den Farbstoff in derselben Konzentration. Die mit den Lösungen ausgeführten Untersuchungen müssen dann identische Ergebnisse liefern.

3. Spektrographische Untersuchung.

Die Absorptionsspektren der isolierten Urochrom-A-Präparate wurden in einem »Gitterspektroskop mit Kamera» der Firma Zeiss untersucht, für die Aufnahme wurden Agfa-Isopan-F.-Platten benutzt. Spezialplatten waren nicht zu bekommen, so dass sich das Wellenlängengebiet, das untersucht werden konnte, auf etwa 370 mμ (untere Grenze des Apparates) bis etwa 620 mμ (obere Grenze der Lichtempfindlichkeit der Platten) beschränkte.

Da die untersuchten Urochrom-A-Lösungen sämtlich dieselbe Extinktionsfähigkeit gegenüber Licht von einer bestimmten Wellenlänge hatten, muss also die Absorptionskurve des ganzen untersuchten Wellenlängenbereiches für sämtliche Lösungen die gleiche sein, wenn die Lösungen identische Farbstoffe enthalten.

In Abb. 27 werden die Absorptionskurven sämtlicher Präparate mit der Absorptionskurve verglichen, die das Präparat *UA-Mensch* liefert. (Die ausgezogenen Kurven entsprechen überall dem *UA-Mensch,* für die übrigen Präparate sind nur die gefundenen Punkte eingetragen.) Es zeigt sich, dass die Kurven der einzelnen Präparate im grossen ganzen zusammenfallen, mit Ausnahme der *UA-Chinon*-Kurve, die einen anderen Verlauf hat. Es sei bemerkt, dass die Abweichungen zwischen den Kurven der aus In-vitro-Versuchen isolierten Präparate und der *UA-Mensch*-Kurve nicht grösser sind als die Abweichungen zwischen den Kurven der einzelnen aus verschiedenen Harnen isolierten Präparate und der Kurve des *UA-Mensch.*

(Aus Raumgründen ist die Kurve für *UA-Polyphen-Meerschw.* in Abb. 27 nicht eingetragen, doch zeigte auch sie eine praktisch vollständige Übereinstimmung mit der Vergleichskurve.)

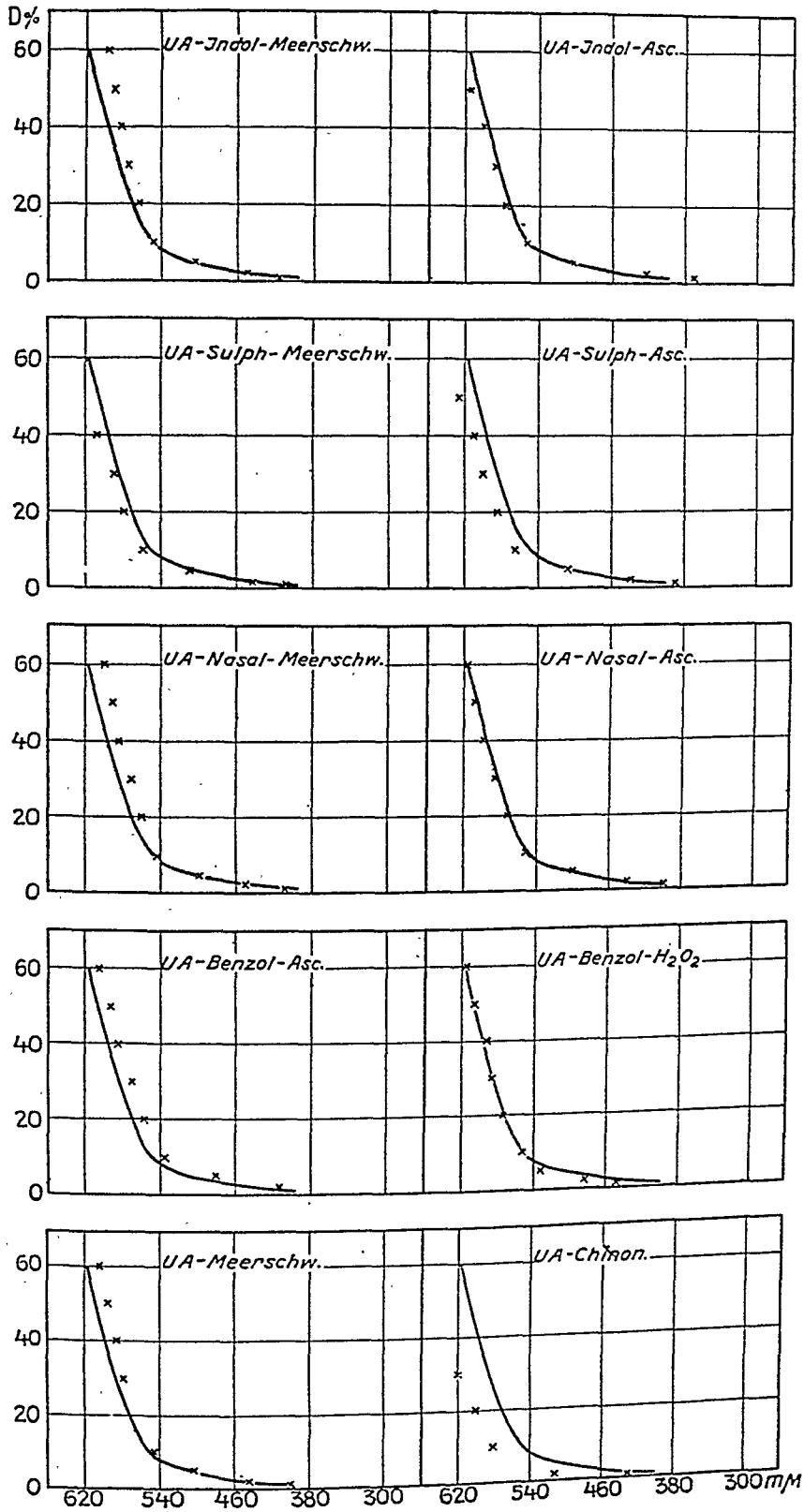

Abb. 27. *Vergleichende spektrographische Untersuchung der Urochrom-A-Präparate.* (Zeiss Gitterspektroskop mit Kamera.)

Es wird die Durchlässigkeit in % und die Wellenlänge in m μ angegeben. Die eingezeichneten Kurven entsprechen überall dem Präparat UA-Mensch, die Punkte (x) dem in jedem Falle zu untersuchenden Präparat. (Eine Durchlässigkeit von 70 % wurde mit sämtlichen Präparaten erst ausserhalb des Anwendungsbereichs der Platten erreicht.)

Bei einigen Präparaten, und zwar sowohl solchen, die aus Harn, als auch solchen, die aus Versuchen in vitro isoliert waren, wurde versucht, durch Zusatz von Salzsäure, Natronlauge oder Ammoniak das Absorptionsspektrum zu beeinflussen. Zwar traten Veränderungen ein, doch waren diese nur quantitativer Art. Die Absorptionsfähigkeit nahm zu und die neuen Absorptionskurven verliefen parallel zu den mit alkoholischen Lösungen erhaltenen.

Es erschien indessen möglich, diese Veränderungen zu weiteren vergleichenden Untersuchungen auszuwerten. Falls es sich zeigen liesse, dass sie bei allen untersuchten Präparaten gleichartig waren, so müsste dies ein Beweis für die Übereinstimmung der isolierten Farbstoffe sein.

Dazu kommt, dass es auch bei der Beurteilung des Wertes der quantitativen Urochrom-A-Bestimmungsmethode von Interesse wäre, wenn man feststellen könnte, dass die unter verschiedenen Verhältnissen gebildeten und als Urochrom A bestimmten Farbstoffe sämtlich in ein und derselben Weise durch die Ammoniak- und Salzsäurezusätze, wie sie das Verfahren vorschreibt, beeinflusst würden.

Für diese Untersuchung quantitativer Veränderungen in der Absorptionsfähigkeit der Lösungen erschien der Pulphrichphotometer am geeignetsten, und zwar unter Verwendung von Filtern, die dem Wellenlängenbereich entsprechen, innerhalb dessen die obige Untersuchung über die Absorptionsspektren ausgeführt worden war.

Die Untersuchung wurde mit sämtlichen Präparaten wie folgt bewerkstelligt:

1. In denselben Urochrom-A-Lösungen, die oben auch verwandt wurden, ist die Extinktion mit sämtlichen Filtern der Reihe S43—S66 bestimmt worden (Schichtdicke 10 mm, 90 % Alkohol als Vergleichslösung).

2. 5 ccm Urochrom-A-Lösung wurden mit 1 ccm 10 % Ammoniak gemischt, und nach 15 Min. wurden die Extinktionswerte abermals bestimmt.

3. 5 ccm der Lösung von 2. wurden mit 2 ccm etwa 10 n HCl gemischt, nach Verlauf von 2 Stunden wurden die Extinktionswerte wie oben bestimmt.

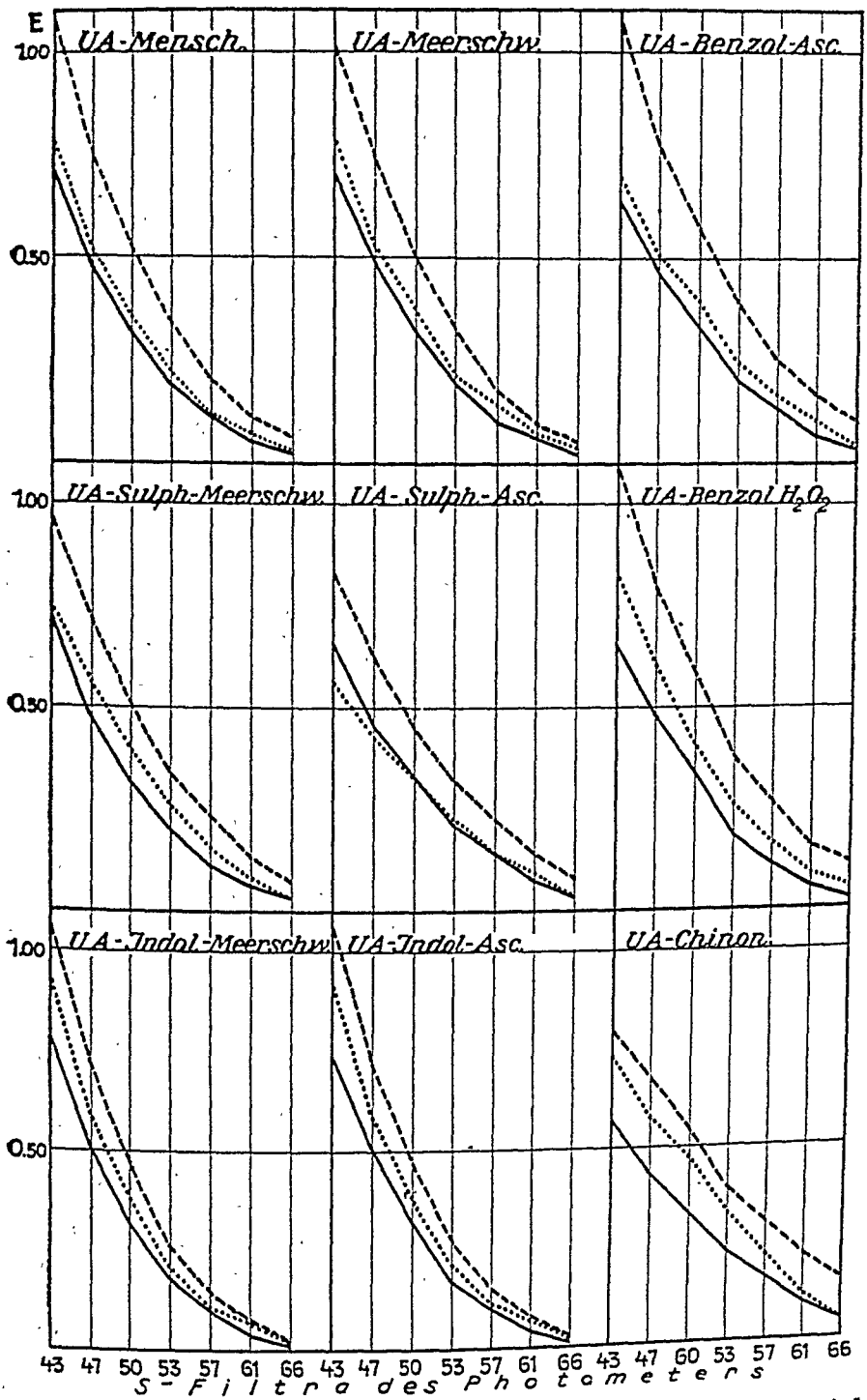

Abb. 28. *Vergleichende Untersuchung über Veränderungen der Extinktionswerte verschiedener Urochrom-A-Präparate bei Zusatz von Ammoniak und Salzsäure mit* ZEISS-*Pulphrichphotometer und den Filtern S47—S66 gemessen.*

Ausgezogene Linien: Alkohollösung. Gestrichelte Linien: Zusatz von Ammoniak. Punktierte Linien: Zusatz von Salzsäure.

Der Zusatz von Ammoniak und Säure bewirkte eine Verdünnung der Urochrom-A-Lösungen, und die nach 2. und 3. erhaltenen Extinktionswerte wurden deshalb alle mit Faktoren multipliziert, welche die durch die Verdünnung bedingte Senkung der Extinktionswerte kompensierten. Bei der Angabe des Ergebnisses entsprechen also alle Extinktionswerte dem ursprünglichen Volumen von 5 ccm.

In Abb. 28 ist das Ergebnis für 9 Präparate angegeben, unter denen sich sowohl aus Harn als aus Lösungen von Versuchen in vitro gewonnenes Urochrom A befindet. Sämtliche Präparate zeigen bei Ammoniakzusatz eine starke Zunahme der Extinktionsfähigkeit. Bei Zusatz von Salzsäure nimmt sie wieder etwas ab, doch bleibt sie (mit Ausnahme für *UA-Sulph-Asc*) höher als für die ursprüngliche alkoholische Lösung. Auch quantitativ herrscht gute Übereinstimmung zwischen den Veränderungen der einzelnen Präparate. Wie in der vorigen Untersuchung zeigt jedoch auch hier *UA-Chinon* einen abweichenden Kurvenverlauf, doch sind die Veränderungen bei Zusatz von Ammoniak und Säure etwa die gleichen wie bei den übrigen Präparaten.

4. Durch Reduktion mit Schwefelwasserstoff lassen sich die isolierten Urochrom-A-Präparate in eine farbschwächere Form überführen.

Bei dem Überblick über das Urochrom-Schrifttum wurde auf den von mehreren Autoren erhobenen Befund hingewiesen, dass Urochrom teils in einer reduzierten, relativ farblosen Form, teils in einer oxydierten, farbstarken Form vorkommen könne. Es schien, als könne eine quantitative Bestimmung dieser Farbschwächung bei der Reduktion beispielsweise mit Schwefelwasserstoff von Wert sein für einen Vergleich zwischen den Eigenschaften der isolierten Präparate.

Der Versuch wurde an sämtlichen Präparaten wie folgt durchgeführt:

5 ccm der früher benutzten alkoholischen Urochrom-A-Lösung wurden mit 10 ccm Aq. dest. und 5 ccm 20 % Metaphosphorsäurelösung versetzt. (Die Metaphosphorsäure wurde deshalb zugesetzt, damit dieselben Lösungen auch für die nachfolgende

TABELLE 56.

Durch Reduktion mit Schwefelwasserstoff lassen sich die isolierten Urochrom-A-Präparate in eine farbschwächere Form überführen.

Für jedes Präparat ist der Extinktionswert nach Behandlung mit Schwefelwasserstoff in Prozent des Extinktionswertes vor der Behandlung angegeben. (Filtrum S51 des PULPHRICH-Photometers.)

Urochrom-A-Präp.	E (% K)	Urochrom-A-Präp.	E (% K)	Urochrom-A-Präp.	E (% K)
UA-Mensch.	77,5	UA-Nasal-Meerschw.	82,1	UA-Nasal-Asc.	74,5
UA-Meerschw.	81,6	UA-Sulph- »	79,5	UA-Sulph- »	65,7
UA-Benzol-Asc.	77,5	UA-Indol- »	81,8	UA-Indol- »	75,0
UA-Benzol-H_2O_2	66,7	UA-Polyphen- »	78,9	UA-Chinon- »	70,0

Untersuchung benutzt werden könnten.) Die Extinktionswerte dieser Lösung wurden mit dem Filter S50 bei einer Schichtdicke von 30 mm bestimmt, worauf 1 Stunde lang ein Schwefelwasserstoffstrom hindurchgeleitet wurde. Die Lösung blieb dann in verschlossenem Glas bis zum nächsten Tag stehen, worauf die Extinktionswertbestimmung wiederholt wurde. In Tabelle 56 ist der Extinktionswert nach der Behandlung mit Schwefelwasserstoff in Prozent des ursprünglichen Wertes angegeben, und es herrscht, wie man sieht, eine relativ gute Übereinstimmung zwischen den verschiedenen Präparaten. (In Kontrollversuchen konnte festgestellt werden, dass wenn dieselben Lösungen gleich lange Zeit standen, doch ohne vorherige Behandlung mit Schwefelwasserstoff, statt dessen eine leichte Erhöhung der Extinktionswerte eintrat.)

5. Die isolierten Urochrom-A-Präparate können nach Behandlung mit Schwefelwasserstoff Dichlorphenolindophenol reduzieren.

Bei der Untersuchung über die Spezifität des Dichlorphenolindophenols als Askorbinsäurereagens im Harn (S. 83) konnte gezeigt werden, dass wahrscheinlich auch Urochrom A das Reagens reduziert. Um diesen früheren Befund zu bestätigen

TABELLE 57.

Urochrom A reduziert Dichlorphenolindophenol.

Titration mit Dichlorphenolindophenol in einer metaphosphorsäurehaltigen Urochrom-A-Lösung, die mit Schwefelwasserstoff behandelt ist. Die Farbe entspricht der Farbe normalen Harns. Das Ergebnis ist in mg% Askorbinsäure ausgedrückt.

Urochrom-A-Präp.	mg % Ask.	Urochrom-A-Präp.	mg % Ask.	Urochrom-A-Präp.	mg % Ask.
UA-Mensch.	0,54	UA-Nasal-Meerschw.	0,68	UA-Nasal-Asc.	0,65
UA-Meerschw.	0,56	UA-Sulph- »	0,48	UA-Sulph- »	0,44
UA-Benzol-Asc.	0,80	UA-Indol- »	0,87	UA-Indol- »	0,30
UA-Benzol-H_2O_2	1,07	UA-Polyphen- »	0,57	UA-Chinon- »	0,68

und zwecks fortgesetzter Vergleiche der Eigenschaften der einzelnen Urochrom-A-Präparate wurde folgender Versuch gemacht:

Durch die gleichen Urochrom-A-Lösungen, die im vorigen Abschnitt zur Untersuchung gelangt waren, wurde 3 Stunden lang Stickstoff hindurchgeleitet, um den Schwefelwasserstoff zu vertreiben, worauf in der gewohnten Weise mit Dichlorphenolindophenol titriert wurde.

Die Farbstärke der Urochrom-A-Metaphosphorsäurelösungen war etwa die gleiche wie die von normalem Meerschweinchenharn, der in Metaphosphorsäure gesammelt worden ist. Um ein ungefähres Bild von den Fehlern bei Bestimmung von Askorbinsäure nach Vorbehandlung mit Schwefelwasserstoff, die durch Urochrom A bedingt sein können, zu geben, ist in Tab. 57 das Titrierergebnis in mg% Askorbinsäure verzeichnet. Aus der Tabelle geht hervor, dass sämtliche Präparate Dichlorphenolindophenol reduziert haben, doch lassen sich gewisse quantitative Schwankungen unterscheiden. *UA-Benzol-H_2O_2* hat ein weit über dem Durchschnitt liegendes Reduktionsvörmigen, während *UA-Sulph-Meerschw.*, *UA-Sulph-Asc.*, *UA-Indol-Asc.* und *UA-Benzol-Asc.* ein schwächeres Reduktionsvermögen aufweisen.

Urochrom-A-Lösungen von gleicher Stärke, die nicht mit Schwefelwasserstoff behandelt sind, vermögen dagegen Dichlorphenolindophenol nicht zu reduzieren.

6. Die isolierten Urochrom-A-Präparate können Phosphormolybdensäure reduzieren.

Bei vorbereitenden Kontrollversuchen nach dem BRIGGSschen Verfahren (S. 87) zeigte es sich, dass Urochrom A Phosphormolybdensäure unter Auftreten einer ähnlichen Farbe wie bei der Reduktion beispielsweise von Hydrochinon durch dasselbe Reagens reduziert.

Auch diese Reaktion, die sich quantitativ verfolgen lässt, wurde für einen Vergleich der Eigenschaften der einzelnen Urochrom-A-Lösungen herangezogen.

Der Versuch wurde mit sämtlichen Urochrom-A-Lösungen wie folgt ausgeführt:

TABELLE 58.

Das Reduktionsvermögen verschiedener Urochrom-A-Präparate gegenüber Phosphormolybdensäure. Bestimmung teilweise nach BRIGGS (S. 86).

Urochrom-A-Präp.	E-Briggs	Urochrom-A-Präp.	E-Briggs	Urochrom-A-Präp.	E-Briggs
UA-Mensch.	0,15	UA-Nasal-Meerschw.	0,33	UA-Nasal-Asc.	0,28
UA-Meerschw.	0,17	UA-Sulph- »	0,16	UA-Sulph- »	0,09
UA-Benzol-Asc.	0,15	UA-Indol- »	0,25	UA-Indol- »	0,08
UA-Benzol-H_2O_2	0,12	UA-Polyphen- »	0,28	UA-Chinon- »	0,135

3 ccm Urochrom-A-Lösung wurden mit 1 ccm etwa 10 n HCl und 5 ccm Wasser versetzt und dann 20 Min. lang im kochenden Wasserbad stehengelassen. Anschliessend wurde wie bei einer gewöhnlichen Polyphenolbestimmung (S. 86) verfahren.

Die gefundenen Extinktionswerte sind in Tab. 58 angegeben, und es zeigt sich, dass sämtliche Präparate Phosphormolybdensäure reduzieren können. Eine gute quantitative Übereinstimmung zeigten die Ergebnisse mit *UA-Mensch, UA-Meerschw., UA-Sulph-Meerschw., UA-Benzol-Asc., UA-Benzol-H_2O_2 und UA-Chinon.*

UA-Nasal-Meerschw., UA-Indol-Meerschw., UA-Polyphen.-Meerschw. und *UA-Nasal-Asc.* bilden eine Gruppe mit bedeutend höherem Reduktionsvermögen, während *UA-Sulph-Asc.* und *UA-Indol-Asc.* ein schwächeres zeigen.

7. Die isolierten Urochrom-A-Präparate lieferten in keinem Falle die Ehrlichsche Diaszoreaktion.

Zusammenfassung der Versuchsergebnisse.

In vergleichenden Untersuchungen sind bestimmte Eigenschaften der in dieser Arbeit erwähnten Urochrom-A-Präparate studiert worden. Diese Präparate sind sowohl aus Harn als aus Lösungen von Versuchen in vitro isoliert.

1. Die Absorptionsspektren der untersuchten Präparate waren, vom Spektrum des *UA-Chinon* abgesehen, praktisch identisch.

2. Sämtliche Präparate wurden bei Zusatz von Ammoniak oder Salzsäure dunkler. Die Zunahme des Extinktionsvermögens wurde quantitativ in verschiedenen Wellenlängenbereichen verfolgt und erwies sich bei sämtlichen Präparaten als etwa gleich gross.

3. Sämtliche Präparate konnten durch Schwefelwasserstoff reduziert werden, und die Reduktion äusserte sich u. a. als eine Schwächung des Extinktionsvermögens. Die Extinktionsabnahme war bei Licht von einer bestimmten Wellenlänge für sämtliche Präparate etwa gleich gross.

4. Sämtliche Präparate vermochten Phosphormolybdensäure sowie nach Behandlung mit Schwefelwasserstoff Dichlorphenolindophenol zu reduzieren. Dabei wiesen jedoch verschiedene Präparate eindeutige quantitative Unterschiede in der Reduktionsfähigkeit auf.

5. Keines der isolierten Präparate lieferte die Ehrlichsche Diazoreaktion.

Besprechung.

Sämtliche Präparate wurden nach einem recht komplizierten Verfahren isoliert, das folgende Eigenschaften des isolierten Produktes voraussetzte: Fällbarkeit mit Kupferazetat, Löslichkeit in Wasser und 90 % Alkohol, Fällbarkeit mit Äther. Die Untersuchung der Absorptionsspektren der einzelnen Präparate ergab, dass diese im grossen ganzen identisch waren. Indessen muss bemerkt werden, dass die Absorptionskurve innerhalb des

untersuchten Wellenlängenbereiches einen gleichmässigen Verlauf ohne charakteristische Maxima oder Minima hatte, weshalb dieses Untersuchungsergebnis keine zu weitgehenden Folgerungen betreffs der Identität der Präparate erlauben dürfte. Die Kurven stimmen aber in so hohem Grade überein, dass die Annahme berechtigt sein wird, dass die untersuchten Farbstoffe eine ähnliche Zusammensetzung haben.

Diese Annahme wird dadurch gestützt, dass sich das Extinktionsvermögen sämtlicher Urochrom-A-Lösungen durch Zusatz von Ammoniak und Salzsäure steigern und durch Reduktion mit Schwefelwasserstoff vermindern liess. Auch die Grösse dieser Veränderungen war bei sämtlichen Präparaten etwa dieselbe.

Die Untersuchungen über das Reduktionsvermögen der einzelnen Präparate gegenüber Phosphormolybdensäure sowie nach Reduktion mit Schwefelwasserstoff gegenüber Dichlorphenolindophenol zeigten indessen so grosse Unterschiede bei den einzelnen Präparaten auf, dass man keine vollständige Identität voraussetzen kann.

Das Ergebnis der Untersuchungen dieses Kapitels liesse sich möglicherweise folgendermassen deuten: Das Urochrom-A-Molekül enthält eine farbige Komponente, die allen untersuchten Präparaten mit Ausnahme des *UA-Chinon* gemeinsam ist. Ausserdem enthält das Molekül eine Komponente von wechselnder Grösse, welche die reduzierenden Eigenschaften bedingt.

Versucht man an Hand der Darstellungsweise der aus den Versuchen in vitro isolierten Urochrom-A-Präparate eine Vorstellung von der Zusammensetzung des Urochroms A zu gewinnen, so ist in erster Linie hervorzuheben, dass ähnliche Farbstoffe bei der Oxydation von Benzol sowohl durch Askorbinsäure als durch Wasserstoffsuperoxyd, und sowohl mit als ohne Zusatz von Eisen erhalten werden konnten. Es kann also im Urochrommolekül weder Eisen noch Askorbinsäure enthalten sein. Wie früher bemerkt, können diese Urochrompräparate auch keinen Stickstoff oder Schwefel enthalten. Beide Grundstoffe werden in der Literatur im allgemeinen als Bestandteile des aus Harn isolierten Urochroms angegeben. In der einleitenden Schrifttumsübersicht wurden indessen Arbeiten referiert, in denen das Vorkommen von Schwefel auf Verunreinigungen der

isolierten Präparate zurückgeführt wurden, und ferner wurde RANGIERS (1935) Befund erwähnt, dass Urochrom sich leicht mit Harnsäure koppeln lasse. Möglicherweise ist der Stickstoffgehalt dadurch zu erklären, dass Urochrom im Harn mit Harnsäure oder anderen stickstoffhaltigen Verbindungen gepaart auftritt.

Urochrom A ist in dieser Arbeit aus Versuchen in vitro mit Benzolderivaten und Indol isoliert worden. Auch Indol enthält einen Benzolkern, und man könnte daher annehmen, dass der Urochrom-A-Bildung Benzol zugrunde läge. In einer früheren Arbeit (EKMAN 1940) konnte indessen gezeigt werden, dass auch Histidin bei Abbau durch Askorbinsäure Urochrom A ergibt. Der Imidazolring des Histidins wird bei diesem Abbau gesprengt, und es erscheint möglich, dass eine Ringspaltung der Bildung des Urochroms A sowohl bei der Oxydation von Benzol als von Imidazol vorausgeht.

Es ist daran zu erinnern, dass LEEDS (1881) bei Oxydation von Benzol durch Wasserstoffsuperoxyd eine Sprengung des Benzolkerns nachweisen konnte.

Der Zweck der hier vorliegenden Untersuchungen über das Urochrom A war, zu zeigen, dass bei der Oxydation zyklischer Verbindungen durch Askorbinsäure oder Wasserstoffsuperoxyd sowohl in vivo als in vitro Farbstoffe entstehen, die wesentliche Eigenschaften gemein haben und die dem alten Begriff des Urochroms A entsprechen dürften, wie es in der auf S. 20 gebotenen Übersicht über die Eigenschaften der früher isolierten Urochrompräparate sich darstellt. Die hier untersuchten Präparate haben im ganzen dieselben Eigenschaften: sie werden in gleicher Weise isoliert wie z. B. DOMBROWSKIS Urochrom und sie haben dieselben Löslichkeits- und Fällbarkeitsverhältnisse. Gemeinsam sind ihnen ferner die reduzierenden Eigenschaften und das Vermögen, sowohl als Urochromogen wie als Urochrom aufzutreten. Die Angaben über das Verhalten zur EHRLICHschen Diazoreaktion variierten; die von mir isolierten Präparate geben diese Reaktion nicht. Frühere Untersucher fanden stets Stickstoff in ihren Präparaten. Gewisse von meinen Präparaten können indessen auf Grund der Darstellungsweise keinen Stickstoff

enthalten. Wie schon früher hervorgehoben, ist es indessen denkbar, dass Urochrom im Harn an stickstoffhaltige Verbindungen gekoppelt vorkommen kann.

Die Urochrom-A-Präparate, die in vitro sowohl aus Verbindungen mit einem Benzolkern wie aus Histidin dargestellt worden waren, ergaben bei Eindampfen der alkoholischen Lösungen sämtlich einen kristallinischen Rückstand. Damit ist die Voraussetzung für eine eingehendere Analyse der Zusammensetzung des in vitro dargestellten Urochroms A gegeben.

Falls die Annahme, dass Urochrom A im Harn an andere Verbindungen gekoppelt vorkommt, richtig ist, dürfte es wahrscheinlich sein, dass man z. B. durch Hydrolyse des Urochroms isoliert aus Harn Präparate darstellen können wird, die ebenfalls in kristallinischer Form erhalten werden könnten.

Fortgesetzte Urochrom-A-Untersuchungen in dieser Richtung dürften indessen auch ein allgemeineres Interesse haben, da sie das Problem des Abbaus des Benzolkerns sowohl in vitro als im lebenden Organismus berühren.

Zusammenfassend dürfte man sagen können, dass die in diesem Kapitel vorgelegten vergleichenden Untersuchungen über verschiedene Farbstoffe, die sowohl aus Harn als aus Lösungen von Versuchen in vitro isoliert worden sind, ergeben, dass diese Farbstoffe dem Urochrom A entsprechen. Man kann annehmen, dass diese Farbstoffe eine gemeinsame, farbige Komponente enthalten, dass aber ihre reduzierenden Eigenschaften durch eine Komponente bedingt sind, die bei verschiedenen Urochrom-A-Individuen verschieden gross ist.

KAP. VIII.

Schlussbemerkungen und Zusammenfassung.

I. Die Askorbinsäurewirkung in vivo.

WACHHOLDER (1942 c) hat darauf hingewiesen, dass sich die Diskussion über die Funktion der Askorbinsäure im Organismus stets hauptsächlich mit zwei Eigenschaften der Askorbinsäure beschäftigt hat, die schon nach den grundlegenden Untersuchungen von SZENT-GYÖRGYI angegeben wurden. »Er hat nämlich 1) gezeigt, dass die Askorbinsäure in der Lage ist, andere Stoffe im reduzierten Zustande zu halten, indem sie vermöge ihres niedrigen Reduktionspotentials durch ihr blosses Vorhandensein dem Gewebssaft ein zerstörende Oxydationen verhinderndes Milieu gibt, und 2) dass die Askorbinsäure selbst reversibel oxydierbar — genauer gesagt zu Dehydroaskorbinsäure dehydrierbar — und wieder zu Askorbinsäure rückreduzierbar ist, sowie dass es für diese Oxydation ein besonderes Ferment, die Askorbinase gibt, mithin dass das Vitamin C in der Lage ist, als Oxydationskatalysator im Gewebsstoffwechsel zu wirken.»

HUSZAK (1937) meinte, die Reduktion von Dehydroaskorbinsäure zu Askorbinsäure könne durch eine Dehydrierung anderer Verbindungen erfolgen, eventuell durch verschiedene Dehydrasen, und die Askorbinsäure trete auf diese Weise als ein Oxydationskatalysator auf.

Die in dieser Arbeit gemachten Feststellungen, dass die Ausscheidung von Polyphenolen bei Zufuhr von Askorbinsäure ansteigt, die Ausscheidung von Urochrom A dagegen abnimmt, lassen sich durch die oben festgestellte reduzierende Fähigkeit der Askorbinsäure erklären.

Dagegen erklärt die oben vorgelegte Anschauung von der Askorbinsäure als einem Oxydationskatalysator nicht die Umwandlung zyklischer Verbindungen. Die von mir gefundene Benzoloxydation ist keine Dehydrierung, und in meinen In-vitro-Versuchen kommt keine Reduktion oxydierter Askorbinsäure vor.

Man muss also nach einer anderen Erklärung für die Wirkung des Vitamins C in diesem Falle suchen, und nach dem, was wir heute über die chemischen Eigenschaften der Askorbinsäure wissen, liegt die Annahme nahe, dass das bei der Oxydation der Askorbinsäure selbst gebildete Wasserstoffsuperoxyd als Oxydationsmittel auftritt. Die ausgeführten Versuche zeigen auch, dass dies in vitro der Fall gewesen sein dürfte.

Es lassen sich gewisse Gründe dafür anführen, dass der Verlauf in vivo der gleiche ist.

1. Die Voraussetzungen dafür, dass Askorbinsäure-Dehydroaskorbinsäure ein reversibles System bilden können, sind vorhanden. Szent-Györgyi (1928) wies das Vorhandensein einer Askorbinase in Pflanzen nach, Wachholder (1942 c) ein askorbinsäureoxydierendes Ferment in den Geweben von Säugetieren. Mehrere Forscher haben Systeme gefunden, welche Dehydroaskorbinsäure zu Askorbinsäure reduzieren.

2. Im Organismus gibt es Peroxydase, durch deren Vermittlung Wasserstoffsuperoxyd als Oxydationsmittel gebraucht werden kann.

3. In den hier vorgelegten Versuchen ist gezeigt worden, dass bei Oxydation von Benzol durch Askorbinsäure oder Wasserstoffsuperoxyd in vitro dieselben Produkte entstehen wie in vivo, nämlich Polyphenole und Urochrom A.

4. Ferner ist es gelungen, durch Zufuhr vegetabilischen Materials den Vitamin-C-Umsatz und die Oxydation einer zyklischen Verbindung im Tierorganismus zu steigern. Diese Aktivierung der Funktion des Vitamins C dürfte darauf beruhen, dass die Vegetabilien Phenole (Benzopyranfarbstoffe) enthalten, die nach Huszak (1937) bei Pflanzen als nichtenzymatische Zwischenkatalysatoren auftreten. Dies setzt nach demselben Autor voraus, dass die Wirkung der Askorbinsäure auf der Bildung von Wasserstoffsuperoxyd beruht hat.

5. Huszak hat ferner gezeigt, dass die Benzopyranfarbstoffe

von Wasserstoffsuperoxyd durch Vermittlung von Peroxydase auch in Anwesenheit von Katalase oxydiert werden können.

Man darf annehmen, dass auch andere zyklische Verbindungen durch das gebildete Wasserstoffsuperoxyd oxydiert werden könnten oder dass sie indirekt durch den oxydierten Benzopyranfarbstoff, der dabei wieder reduziert wird, oxydiert werden. Möglicherweise liegt die Bedeutung der Benzopyranfarbstoffe gerade darin, dass ihre Oxydation durch Peroxydase/Wasserstoffsuperoxyd so schnell verläuft, dass anwesende Katalase die Reaktion nicht hemmt, was dagegen vielleicht bei einer direkten Oxydation anderer zyklischer Verbindungen der Fall sein würde.

6. In einem Falle hat gezeigt werden können, dass Wasserstoffsuperoxyd, das in einer primären Oxydation gebildet worden ist, durch Vermittlung von Katalase für eine sekundäre, gekoppelte Reaktion herangezogen werden kann. HOLMBERG (1939) konnte nämlich durch Verwendung einer reindargestellten Urikase die von KEILIN und HARTREE (1936) gemachte Annahme bestätigen, dass die gekoppelte Oxydation von Alkohol im System Urikase-Urinsäure durch das gebildete Wasserstoffsuperoxyd nur bei Anwesenheit von Katalase stattfände.

Es dürfte indessen heute allgemein die Ansicht bestehen, dass Wasserstoffsuperoxyd in den meisten Dehydrogenasesystemen gebildet wird, und wahrscheinlich werden die dabei gebildeten Mengen bedeutend grösser sein als die bei Oxydation der Askorbinsäure anfallenden. Dass man trotzdem eine Beeinflussung der Umwandlung zyklischer Verbindungen durch Änderungen in der Askorbinsäurezufuhr erzielen kann, mag vielleicht folgende Ursachen haben:

Die Umwandlung zyklischer Verbindungen kann auf gewisse Organe beschränkt sein, in denen eine Anreicherung von Askorbinsäure und zyklischen Verbindungen erfolgt, und in denen das Verhältnis Peroxydase/Katalase günstig ist.

Nach THEORELL (1943) entsteht Wasserstoffsuperoxyd u. a. »durch eine Reaktion zwischen Peroxydase, O_2 und Dioxymaleinsäure. Es reichert sich aber nicht in der Lösung an, sondern der Überschuss wird, je nachdem er gebildet wird, in einer

echten, sekundären, peroxydatischen Reaktion verbraucht.» Das Wasserstoffsuperoxyd wird also in demselben System verbraucht, in welchem es gebildet wird, und möglicherweise ist dies der übliche Verlauf, während das bei der Oxydation der Askorbinsäure gebildete Wasserstoffsuperoxyd vielleicht für andere Reaktionen verfügbar wird.

II. Zusammenfassung.

Als Hauptergebnisse der hier vorgelegten Untersuchungen dürfte folgendes gelten können:

1. Als Ergebnis einer Literaturübersicht wird hervorgehoben, dass bei der gegenwärtigen Einsicht in die chemische und pharmakologische Funktion des Vitamins C diese Funktion grossenteils als eine Umwandlung oder Stabilisierung zyklischer Verbindungen aufgefasst werden kann, sowie dass die Entstehung von Urochrom A in Zusammenhang sowohl mit Vitamin C als mit zyklischen Verbindungen gebracht werden kann. (Kap. I.)

2. Es sind neue quantitative Bestimmungsmethoden für Urochrom A, Salizylsäure und Indol ausgearbeitet worden. Die quantitative Bestimmung von Askorbinsäure im Harn ist zum Gegenstand einer experimentellen Untersuchung gemacht worden. (Kap. II.)

3. Zyklische Verbindungen werden in vitro und in vivo durch Askorbinsäure oxydiert. Das besagt, dass Askorbinsäure für das Entgiftungsvermögen des Organismus von Bedeutung ist, und es wird angenommen, dass man darin eine wesentliche Funktion des Vitamins C zu erblicken hat. (Diese Funktion wird indessen durch Zufuhr sehr grosser Mengen von Askorbinsäure gehemmt.)

Frühere Untersuchungen über den Antagonismus zwischen verschiedenen Hormonen und Askorbinsäure, sowie die Entgiftung gewisser zyklischer Verbindungen durch Askorbinsäure in vivo und in vitro werden vor dem Hintergrunde der hier vorgelegten Ergebnisse besprochen. (Kap. III und IV.)

4. Die Oxydation zyklischer Verbindungen in vitro durch Askorbinsäure lässt sich dadurch erklären, dass bei der Oxyda-

tion der Askorbinsäure selbst Wasserstoffsuperoxyd entsteht, das als Oxydationsmittel auftritt. (Kap. V.)

Es werden mehrere Gründe dafür angezogen, dass der Verlauf in vivo der gleiche sein dürfte. Möglicherweise ist Askorbinsäure ein Bestandteil eines gleichartigen Oxydationssystems, wie es HUSZAK für die Peroxydasepflanzen angegeben hat, das im übrigen aus einer Askorbinsäureoxydase, einer Peroxydase, einem nichtenzymatischen Zwischenkatalysator sowie einem System besteht, das die gebildete Dehydroaskorbinsäure zu Askorbinsäure reduzieren kann. (Kap. VI und VIII.)

5. Die Aufnahme von Vegetabilien, die wahrscheinlich in dem vorgenannten Oxydationssystem enthaltene nichtenzymatische Zwischenkatalysatoren (Benzopyranfarbstoffe oder andere Phenole) führen, steigert im Tierorganismus den Umsatz an Vitamin C sowie die Fähigkeit der Askorbinsäure, zyklische Verbindungen zu oxydieren.

Damit hat mit der »Oxydation zyklischer Verbindungen durch Askorbinsäure» als Test das Vorhandensein von Faktoren festgestellt werden können, welche die Wirkung der Askorbinsäure steigern, und auch der wahrscheinliche Mechanismus ihrer Funktion ist damit angegeben. (Kap. VI.)

6. Bei der Oxydation zyklischer Verbindungen durch Askorbinsäure entstehen Oxydationsprodukte, die ohne Einwirkung von Askorbinsäure weiteroxydiert werden und dann Farbstoffe liefern, die dem Begriff des Urochroms A entsprechen. Ist indessen Askorbinsäure im Überschuss anwesend, so wird diese Weiteroxydation und damit auch die Urochrom-A-Bildung gehemmt. (Kap. III, IV und VII.)

Wahrscheinlich handelt es sich hier um eine Gruppe eng miteinander verwandter Stoffe, die wesentliche Eigenschaften gemein haben, jedoch nicht identisch sind. (Kap. VII.)

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VOL. 8. SUPPLEMENTUM XXIII

AUS DEM ZENTRALLABORATORIUM DES SAHLGRENSCHEN KRANKENHAUSES IN GÖTEBORG, SCHWEDEN

DIE WIRKUNG VON BENZOESÄURE UND VERWANDTEN STOFFEN AUF DIE AMMONIAKBILDUNG IM NIEREN- UND LEBER-GEWEBE VON RATTEN

VON

BIRGER HERNER

GÖTEBORG

1 9 4 4

VORWORT.

Die vorliegende Arbeit wurde im Zentrallaboratorium des Sahlgrenschen Krankenhauses in Göteborg durchgeführt.

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Göteborg, den 1. April 1944.

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Problemstellung.

Die vorliegende Arbeit beabsichtigt, den Einfluss der Benzoesäure, der Mandelsäure und den einiger nahe verwandter Stoffe auf die Ammoniakbildung vor allem im Nieren- und Leber-Gewebe von Ratten zu untersuchen.

Die Vermutung eines Einflusses auf die Ammoniakbildung in den genannten Geweben entstand zunächst im Hinblick auf die *Mandelsäure*, und zwar tauchte sie beim *Vergleich einiger experimentellen und klinischen Beobachtungen* auf. LEHMANN (1938) hatte nämlich nachgewiesen, dass Mandelsäure die Fähigkeit gewisser Hefezellen, Milchsäure zu oxydieren, hemmt. Da sowohl Milchsäure als auch Mandelsäure α-Oxysäuren sind, und da die Oxydation der Milchsäure durch einen Angriff des Enzyms am α-Kohlenstoffatom eingeleitet wird, wäre es denkbar, dass die Hemmung eine sogenannte konkurrierende Enzymhemmung wäre, bei der ein Teil des Enzyms von der in diesem Punkte gleichgebauten Mandelsäure engagiert würde. Analog wäre es auch denkbar, dass die Mandelsäure die Aminosäuredesaminierung hemmt, denn es ist gezeigt worden, dass wenigstens bei gewissen Aminosäuren die Oxydation beim substituierten α-Kohlenstoffatom eingeleitet wird.

Diese auf experimentelle Untersuchungen gegründete Vermutung kann durch einige klinische Beobachtungen gestützt werden. In einigen Fällen, wenn Mandelsäure als Harnwegantiseptikum verwendet worden war, schien es nämlich, als hemme sie die Ammoniakabwehr, die der Organismus zwecks Kompensation der durch die Säure hervorgerufenen Acidose mobilisiert. NIELSEN (1941) beobachtete so in einem Fall eine starke Entkalkung des Skelettes bei einer Gravida nach 3—4-monatiger Behandlung mit Mandelsäure und Salmiak. Dies kann darauf deuten, dass die Ammoniakproduktion des Organismus insuffizient war, weshalb zur Neutralisation fixe Alkalien herangezogen werden mussten. ODIN (persönliche Mitteilung) beobachtete ferner eine erstaunlich geringe Ammoniakproduktion im Harn bei einem Cystopyelitisfall, der intensiv mit Man-

delsäure behandelt wurde. Die referierten klinischen Beobachtungen sind freilich an und für sich durchaus keine einwandfreien Argumente für die Annahme, dass Mandelsäure die Ammoniakbildung in der Niere hemme, aber zusammen mit dem Ergebnis der experimentellen Untersuchung werden sie alle zu Indizien, die auf die Möglichkeit einer gemeinsamen Erklärung hindeuten.

Ausgehend von den referierten Untersuchungen, machte ich, HERNER (1942), mit Hilfe der Warburgschen Technik einige Versuche über die Einwirkung der Mandelsäure auf den Sauerstoffverbrauch und die Ammoniakbildung im Nieren- und Leber-Gewebe von Ratten. Auch hierbei ergab sich unter anderm als wahrscheinlich, dass Mandelsäure die spontane Ammoniakbildung in diesen Geweben hemmt.

Indessen spricht auch manches dafür, dass nicht bloss die Mandelsäure, sondern auch die ihr nahe verwandte Benzoesäure die Ammoniakbildung in Niere und Leber hemmt. Verschiedene Forscher haben nämlich durch *In-vitro-Versuche* nachgewiesen, dass *Benzoesäure im Nieren- und Leber-Gewebe die Oxydation verschiedener Stoffe hemmt.* So wiesen JOWETT u. QUASTEL (1935 a, b) eine Hemmung der Oxydation von Buttersäure und Krotonsäure im Leberschnitt von Meerschweinchen und von Buttersäure im Leberschnitt von Ratten nach. QUASTEL u. WHEATLEY (1935) wiesen eine Hemmung der Oxydation von Acetessigsäure im Nierenschnitt von Meerschweinchen und Ratten nach, GRIFFITH (1937) eine Hemmung der spontanen Oxydation in Nieren- und Leber-Schnitten von Ratten, und KLEIN u. KAMIN (1941) eine Hemmung der Oxydation einiger d-Aminosäuren in Nieren- und Leber-Gewebe von Ratten und bei Versuchen mit gereinigtem d-Aminosäureoxydaseenzym.

Es scheint somit, als greife die Benzoesäure hemmend in verschiedenerlei Reaktionen des intermediären Stoffwechsels in Niere und Leber ein. Dies kann daher Anlass zu der Vermutung geben, sie hemme auch die Bildung von Ammoniak in diesen Organen, die vor allem beim Aminosäureabbau zustande kommen dürfte — eine Frage, die später näher berührt werden soll.

Zeigt sich somit, dass Benzoesäure die gleiche Hemmung wie Mandelsäure auslöst, muss die oben skizzierte hypothetische Erklärung aufgegeben werden, die in der Annahme bestand, die durch Mandelsäure verursachte Hemmung beruhe auf einer Enzymkonkurrenz, hervorgerufen durch die dort besprochene, in gewisser Hinsicht

ähnliche chemische Konstitution der Mandelsäure und der α-Aminosäuren.

Um diese Fragen zu klären, zielt die *vorliegende Arbeit* auf einen *Vergleich zwischen dem Einfluss der Mandelsäure und dem der Benzoesäure auf die Ammoniakbildung im Nieren- und Leber-Gewebe* ab. Daneben werden einige in ungleicher Weise chemisch verwandte Stoffe untersucht, um womöglich *die aktive Gruppe* erkennen zu können. Ferner wird die Wirkung auf verschiedene ammoniakbildende Prozesse je für sich untersucht, in der Absicht, zu erfahren, ob die *Giftwirkung auf* die *verschiedenen Enzymsysteme* die gleiche ist. Weiterhin werden einige Versuche mit *Bacterium coli* unternommen, um zu untersuchen, ob von den nachgewiesenen Verhältnissen etwa einiges auch für den Stoffwechsel der Bakterien Geltung hat.

Die ursprünglich mehr klinisch gerichtete Problemstellung betreffend die Hemmung der Ammoniakabwehr des Organismus durch Mandelsäure wird also zurücktreten hinter einer experimentellen Untersuchung über den Einfluss der Mandelsäure, der Benzoesäure und denjenigen einiger mit diesen nahe verwandter Stoffe auf verschiedene ammoniakbildende Prozesse. Durch ein derartiges Studium der Wirkung dieser Stoffe auf verschiedene intermediäre Abbauprozesse je für sich dürften bessere Voraussetzungen für ein vertieftes Verständnis für sowohl deren Giftwirkung auf höhere und niedrigere Organismen als auch für ihre therapeutische Wirkung geschaffen werden. In diesem Zusammenhang sei betont, dass ausser der Benzoesäure und der Mandelsäure besonders noch die Salicylsäure grosses Interesse verdient, da sie, jenseits ihrer wichtigen therapeutischen Verwendung, wie die Benzoesäure in der Lebensmittelchemie eine bedeutende Rolle als Konservierungsmittel spielt.

Frühere Untersuchungen über den Einfluss der Benzoesäure und nahverwandter Stoffe auf verschiedene Enzymsysteme besonders innerhalb des intermediären Stoffwechsels.

Über die therapeutischen Wirkungen der Mandelsäure und der Salicylsäure liegt eine beträchtliche Anzahl klinischer Abhandlungen vor, die jedoch hier nicht besprochen werden sollen. Ebensowenig sei hier auf die zahlreichen allgemeinen Untersuchungen eingegangen, die hinsichtlich der *Desinfektionswirkung der Benzoesäure und der Salicylsäure* vorgenommen wurden, es soll vielmehr nur auf einige Arbeiten mit Literaturangaben hierüber verwiesen werden: SUTER (1941), betreffend die Salicylsäure und ihre Derivate, und ANDRESEN (1929) sowie LAGONI (1940/41) hinsichtlich der Benzoesäure.

Dagegen ist es von Interesse, hier über einige frühere Untersuchungen zu referieren, die sich mit ähnlichen Problemen befassen, wie sie die vorliegende Arbeit behandelt, nämlich mit dem speziellen *Einfluss* der betreffenden Stoffe *auf verschiedene Enzymsysteme.*

Es gibt einige Untersuchungen über den *Einfluss der Benzoesäure* und der *Salicylsäure* auf verschiedene *beim Abbau von Nährstoffen wirksame Enzyme.* Die Ergebnisse dieser Untersuchungen scheinen jedoch nicht ganz übereinzustimmen. Immerhin scheint aus ihnen hervorzugehen, dass Benzoesäure die *Pepsinverdauung* hemmt (KOENIG 1931, KLUGE 1933). Während nach KLUGE (1933) die stärke- und fett-verdauenden Enzyme nicht beeinflusst zu werden scheinen, finden BLEYER, DIEMAIR u. LEONHARD (1933) eine Hemmung der Wirkung von sowohl *Pepsin* als auch von *Amylase* und *Lipase* durch Benzoesäure. Im allgemeinen übt nach diesen Forschern Salicylsäure eine noch kräftigere Wirkung aus als Benzoesäure, während ihnen Natriumbenzoat eine erheblich schwächere Wirkung als freie Benzoesäure zu haben scheint, auch wenn bei den entsprechenden Versuchen pH auf gleichem Niveau gehalten wird.

Im Hinblick auf die vorliegende Untersuchung ist indessen der *Einfluss* der betreffenden Stoffe *auf die Wirksamkeit der intracellularen Enzyme* von grösserem Interesse. Über einige Arbeiten wurde schon berichtet. Sie zeigten, dass Benzoesäure den Abbau von sowohl gewissen *Fettsäuren* als auch gewissen *d-Aminosäuren im Nieren- und Leber-Gewebe* hemmt. Gerade die Hemmung des Abbaus von d-Aminosäuren ist hier von Bedeutung. KLEIN u. KAMIN (1941), die dies untersucht haben, studierten unter anderem die Hemmungswirkung auf d-Aminosäureoxydaseenzym von hohem Reinheitsgrad und fanden, dass die durch die Benzoesäure hervorgerufene Hemmung reversibel ist und wahrscheinlich auf der Bildung eines Benzoesäure- d-Aminosäureoxydase-Komplexes beruht.

Weiterhin ist es interessant, dass THUNBERG (1913) gezeigt hat, dass *Benzoesäure* und verschiedene ihr nahestehende Stoffe in überlebender *Froschmuskulatur* den *Gasaustausch* stark *hemmen*, sowie dass MÅHLÉN (1928) gezeigt hat, dass *Benzoesäure sowie o-Oxy-, m-Oxy- und p-Oxy-Benzoesäure die Succinodehydrogenase* in der Muskulatur *hemmen*, weiterhin auch, dass COLLETT u. CLARKE (1929) nachgewiesen haben, dass *Benzoesäure* die Wirksamkeit der *Citronensäure-, Glycerinphosphorsäure-, Milchsäure-, l-Äpfelsäure- und Succino-Dehydrogenase* in der Muskulatur *hemmt*. BLEYER u. MITARB. (1933) zeigten in der oben zitierten Untersuchung auch, dass *Benzoesäure und Salicylsäure* die Wirkung der *Succinodehydrogenase* in der Muskulatur und die der *Schardingerdehydrase* in der Milch *hemmen*. Auch hier ist die Wirkung des Natriumbenzoats geringer als die der freien Benzoesäure. GRIFFITH (1937) fand, dass die durch Benzoesäure hervorgerufene Hemmung des Stoffwechsels im Lebergewebe mehr in den *Glykose-Milchsäure-Stoffwechsel* als in den Bernsteinsäurestoffwechsel *eingreift*. IVÁNOVICS (1942) konnte zeigen, dass *Natriumsalicylat das Wachstum gewisser Bakterien*, unter anderm das von *Colibakterien, hemmt*. Bei näherer Untersuchung fand er, dass die Salicylsäure in den Bakteriencellen die *Bildung der* für deren Wachstum nötigen *Pantothensäure hemmt*. Bei der Untersuchung von mit der Salicylsäure verwandten Stoffen fand er, dass die Benzoesäure eine schwächere und weniger typische Wirkung ausübt.

Nachdem vorliegende Untersuchung begonnen worden und die vorläufige Mitteilung über die Hemmungswirkung der Mandelsäure herausgekommen war (HERNER 1942), wurde von v. EULER und

MITARB. eine Anzahl von Untersuchungen über die *hemmende Wirkung der Salicylsäure* und einiger nahverwandter Stoffe auf verschiedene *isolierte Enzymsysteme* veröffentlicht. Diese Arbeiten gehen aus von den obengenannten Untersuchungen von IVÁNOVICS sowie von der Entdeckung des *Antagonismus zwischen der p-Aminobenzoesäure und den Sulfanilamidderivaten* durch WOODS u. FILDES (1940). v. EULER und MITARB. vermuteten, dass durch Untersuchungen an isolierten Enzymsystemen ein klarerer und übersichtlicherer Bescheid darüber zu erhalten sei, wo die Antivitamine angreifen, als auf dem Wege der früheren Untersuchungen an in den Cellen gebundenen Enzymsystemen. Die Verfasser (ADLER, v. EULER u. SKARZYNSKI 1942; v. EULER 1942) begannen mit der Untersuchung der *Glykose- und Milchsäure-Dehydrasewirkung*. Die Cozymase ist ein Bestandteil dieser Enzyme. Als Hemmungssubstanzen, Antivitamine, verwendeten sie in erster Linie Nikotinsäure und α-Pyridinsulfonsäure, die mit der Cozymase chemisch verwandt sind. Diese Stoffe hemmen die Wirkung der untersuchten Enzymsysteme, was als eine Verdrängung der Cozymase vom Apoenzym erklärt werden könnte, analog dem Konkurrenzverhältnis, das man als zwischen der p-Aminobenzoesäure und den Sulfanilamidderivaten bestehend annimmt. Gegen diese Erklärung spricht jedoch, nach den Verfassern, dass Stoffe ohne deutliche chemische Verwandtschaft mit der Cozymase, z. B. verschiedene *cyclische Carbonsäuren und Sulfonsäuren*, wie Benzoesäure, Salicylsäure und Sulfanilsäure ebenfalls die untersuchten *Enzymsysteme hemmen*.

Bei Milchsäuredehydrase findet v. EULER (1942) folgende Hemmung, verursacht durch verschiedene, bei den angegebenen Konzentrationen von ihm untersuchte Stoffe:

0,02	Mol/l	Benzoesäure	24 %
0,016	Mol/l	Benzamid	22,5 %
0,02	Mol/l	Salicylsäure	72 %
0,04	Mol/l	»	84 %
0,02	Mol/l	Sulfanilsäure	38 %

v. EULER u. AHLSTRÖM (1943 a) erweiterten die Untersuchungen und fanden, dass Salicylsäure auch die *Succinodehydrasewirkung hemmt*. Dies, meinen die Verfasser, spreche wiederum gegen den Gedanken, dass die Hemmungswirkung der untersuchten Stoffe auf einer Verdrängung der Cozymase beruhe, da die Succinodehydrase,

wie bekannt, ohne Cozymase arbeitet. DIESELBEN FORSCHER (1943 b) konnten an lebenden Ratten auch nachweisen, dass der Gehalt an Brenztraubensäure im Blut nach Salicylatzufuhr per os steigt, was nach Ansicht der Verfasser auf einer Hemmung der Enzymwirkung beruht.

Aus ihren Untersuchungen ziehen v. EULER u. AHLSTRÖM (1943 b) den Schluss, dass die Hemmungswirkung der Salicylsäure auf die untersuchten Enzymsysteme wahrscheinlich nicht auf einer Coenzymverdrängung vom Apoenzym beruht, sondern darauf, dass die Salicylsäure in der Apozymase eine andere Gruppe angreift als die Affinitätsstelle der Cozymase, oder dass sie, weniger spezifisch, die ganze Apozymase schwächt.

Diese Untersuchungen von v. EULER u. MITARB. sind von besonderem Interesse, teils weil sie sich mit der Hemmungswirkung verschiedener Stoffe befassen, die auch in der vorliegenden Arbeit eben darauf untersucht wurden, teils weil sich *Berührungspunkte zwischen* den *hier und dort untersuchten Enzymsystemen* finden lassen. So kann die *l* (+) *Glutaminsäure,* die hier untersucht wurde, durch eine Dehydrase abgebaut werden, die in ihrer Wirkung von Codehydrase I (Cozymase) oder Codehydrase II abhängig ist (v. EULER, ADLER, GÜNTHER u. DAS 1938). Für die in der vorliegenden Arbeit untersuchten Verhältnisse gilt jedoch, dass die Enzyme sich oft noch in den Zellen befinden und nie so frei sind wie bei den Versuchen v. EULERS und seiner MITARB.; dennoch können sich die Ergebnisse hier und dort gegenseitig beleuchten, weshalb noch eine diesbezügliche Diskussion erfolgen soll.

Jedenfalls scheint sich aus den referierten Untersuchungen zu ergeben, dass *Benzoesäure und Salicylsäure verschiedene Prozesse im intermediären Stoffwechsel vergiften.* Hinsichtlich einer entsprechenden Wirkung der Mandelsäure scheinen ausser den bereits angeführten Untersuchungen von LEHMANN (1938) und HERNER (1942) keine weiteren vorzuliegen.

Ammoniakbildende Prozesse im Nieren- und Leber-Gewebe.

Da die vorliegende Untersuchung beabsichtigt, die Wirkung der Benzoesäure und nahe verwandter Stoffe auf die Ammoniakbildung im Nieren- und Leber-Gewebe zu studieren, sollen die wichtigsten ammoniakbildenden Prozesse in Niere und Leber und die dabei arbeitenden Enzyme einleitend kurz besprochen werden.

Ammoniakbildung aus Aminosäuren.

Seit langer Zeit ist es bekannt, dass das Ammoniak, das in den ebengenannten Organen gebildet wird, zum grossen Teil bei der *Desaminierung der Aminosäuren* freiwird. Indessen waren es im weiteren vor allem die ganz besonders eingehenden Untersuchungen von Krebs (1932, 1933 a und b, 1935 a und b, 1939) über den Abbau der meisten bekannten Aminosäuren im Nieren- und Leber-Gewebe, durch die unsere Kenntnisse von der Rolle der Aminosäuren als der wichtigsten Ammoniakspender in Niere und Leber vertieft wurden. Auch Bernheim u. Mitarb. (1932, 1934 a und b, 1935 a und b, 1936) haben hierüber wichtige Untersuchungen gemacht.

In den folgenden Untersuchungen wird daher der Einwirkung der Benzoesäure und nahe verwandter Stoffe auf die Desaminierung der Aminosäuren im Nieren- und Leber-Gewebe besondere Aufmerksamkeit zugewandt.

Wie bekannt sind *die in der Natur vorkommenden Aminosäuren* zum weitaus grössten Teil von *l-Konstitution*, weshalb es am nächsten liegt, vor allem l-Aminosäuren zu untersuchen. Es ist jedoch wichtig, auch die *d-Aminosäuren* zu studieren, denn mancherlei spricht dafür, dass auch sie *beim Stoffwechsel eine Rolle spielen* können. Einige der wichtigsten Untersuchungen, die dies beleuchten, sind die folgenden:

Wohlgemuth (1905) fand bei der Fütterung von Kaninchen mit der dl-Form von Tyrosin, Leucin, Asparaginsäure und Glutamin-

säure, dass die l-Komponente so gut wie vollständig abgebaut wurde, während die d-Komponente in grösserem oder kleinerem Umfang im Harn ausgeschieden wurde. DAKIN (1910/11) und KOTAKE, MATSUOKA u. OKAGAWA (1922) konnten das Gleiche bei Verfütterung von dl-Tyrosin an Katzen resp. Kaninchen nachweisen, ebenso ABDERHALDEN u. TETZNER (1935) bei Versuchen mit dl-Alanin an, unter anderem, Ratten. Aus diesen Versuchen geht hervor, dass der Totalorganismus l-Aminosäuren leicht, d-Aminosäuren dagegen nur ziemlich begrenzt abbauen kann.

Indessen liegen auch Versuche vor, aus denen sich ergibt, *dass der Organismus d-Aminosäuren nicht nur abbauen, sondern* sie *sich* sogar *zunutze machen kann.* Mehrere Forscher haben so durch Tierversuche zeigen können, dass die d-Form einer unentbehrlichen Aminosäure das Wachstum bei Tieren im Gang halten kann. Von BERG u. POTGIETER (1931/32), DU VIGNEAUD, SEALOCK u. VAN ETTEN (1932) wurden derartige Versuche mit Tryptophan angestellt, mit Methionin von JACKSON u. BLOCK (1932/33) und mit Histidin von COX u. BERG (1934). CONRAD u. BERG (1937) konnten weiterhin nachweisen, dass Versuchstiere, die auf l (—)histidinarme, aber mit d (+)Histidin komplettierte Kost gesetzt waren, in reichlicherer Menge l (—)Histidin im Gewebe aufspeicherten, als es den kleinen, in der Nahrung vorhandenen Mengen entsprechen konnte. Die direkte Umwandlung einer d-Aminosäure in die entsprechende l-Form konnten DU VIGNEAUD u. IRISH (1935 u. 1937/38) zeigen, indem sie bei Tierversuchen die d-Komponente der biologisch nicht vorkommenden Phenylaminobuttersäure per os verabreichten, worauf sie nachweisen konnten, dass im Harn das Acetylderivat der l-Komponente der Aminosäure ausgeschieden wurde.

In den letzten Jahren ist es auch geglückt, unter Verwendung von *Isotopen* einen etwas tieferen *Einblick in diese Umwandlung von d-Aminosäuren* in l-Aminosäuren zu bekommen. So haben RATNER, SCHOENHEIMER u. RITTENBERG (1940) nach Fütterung von Ratten mit d (+) Leucin, das an der Kohlenstoffkette Deuterium als Indikator und in der Aminogruppe schweren Stickstoff (N^{15}) als Indikator enthielt, nachweisen können, dass die d-Komponente vom Organismus abgebaut und teilweise in l (—)Leucin umgewandelt wird. Nach Isolierung dieser Aminosäure vom Gewebeeiweiss konnte eine beträchtliche Menge Deuterium an der Kohlenstoffkette nachgewiesen werden, was ein Zeichen dafür war, dass sie

zu einem grossen Teil von der d-Komponente herstammte. Die Aminogruppe enthielt dagegen nur eine geringe Menge N^{15}, wie sie auch in anderen Aminosäuren nachgewiesen werden konnte. Die Verfasser nehmen daher an, dass die Inversion der d-Form in die l-Form in zwei Etappen stattfindet, zuerst handle es sich um eine vollständige Desaminierung und danach um eine Aminierung, wobei Stickstoff auch von anderen Aminosäuren genommen werde, wodurch die Menge isotopen Stickstoffs verhältnismässig klein wird. Bei Versuchen mit d(—)Lysin konnten RATNER, WEISSMANN u. SCHOENHEIMER (1942/43) mittels gleicher Versuchstechnik einen gewissen Abbau dieser Aminosäure nachweisen, indem N^{15} in verschiedenen aus dem Gewebeeiweiss stammenden Aminosäuren sowie im Harnammoniak und im Harnstoff wiedergefunden werden konnte, wohingegen eine Inversion in die l-Komponente nicht nachweisbar war.

Lange war man der Meinung, die in der Natur vorkommenden Aminosäuren zeigten ausschliesslich die l-Konstitution, und bei den oben referierten Untersuchungen handelte es sich bei den dazu verwendeten d-Aminosäuren auch nur um synthetisch hergestellte. Es ist daher interessant, dass es in mehreren Fällen gelungen ist, die *Bildung von d-Aminosäuren in natürlichen Geweben* nachzuweisen. So geben JACOBS u. CRAIG (1935) das Vorkommen der d-Komponente des Prolins im Hydrolysat des Ergotinins und einigen verwandten Alkaloiden und IVÁNOVICS u. BRUCKNER (1937) das der d-Komponente der Glutaminsäure in der Kapselsubstanz bei Milzbrandbazillen und einigen verwandten Mikroorganismen an, während LIPMANN, HOTCHKISS u. DUBOS (1941) nachwiesen, dass gewisse vom Bac. brevis, der mit den ebengenannten Mikroorganismen verwandt ist, herstammende Peptide zum Teil aus d-Aminosäuren bestehen. KÖGL u. ERNLEBEN (1939) behaupten ferner das Vorkommen verschiedener d-Aminosäuren, im besonderen von d(—)Glutaminsäure, im Gewebeeiweiss maligner Tumoren. Diesbezüglich ist eine äusserst lebhafte Diskussion entstanden, und es dürfte einstweilen noch nicht feststehen, ob es mit diesem Befunde seine Richtigkeit hat. Viele Gründe scheinen dafür zu sprechen, dass im intakten Tumorengewebe keine d-Aminosäuren vorkommen, sondern dass sie erst während der Analyse durch Racemisierung aus entsprechenden l-Aminosäuren entstehen (siehe Zusammenfassung von SCHOENHEIMER u. RATNER, 1941).

Im Hinblick auf das zuvor Gesagte scheint es aber jedenfalls nicht mehr adäquat zu sein, die d-Aminosäuren als »nicht natürlich» zu bezeichnen. Freilich kommen sie, soviel man bis jetzt weiss, in der Natur so sparsam vor, dass sie beim Stoffwechsel nur eine sehr geringe Rolle spielen können; der Tatsache aber, dass sie vorkommen, ist beim Studium der Fähigkeit des Organismus, Aminosäuren abzubauen, natürlich Beachtung zu schenken.

Aus vorher referierten Untersuchungen ging klar hervor, *dass der Organismus auch d-Aminosäuren abzubauen vermag;* es besteht aber eine gewisse Verschiedenheit zwischen dem durch den Totalorganismus bewirkten Abbau von d-Aminosäuren und deren Abbau bei In-vitro-Versuchen mittels aus Nieren- und Leber-Gewebe stammenden d-Aminosäureoxydaseenzyms. Im Totalorganismus werden die d-Aminosäuren bedeutend langsamer als die l-Aminosäuren abgebaut, während in vitro das Gegenteil der Fall ist. Die erwähnte *begrenzte Fähigkeit des Totalorganismus, d-Aminosäuren abzubauen,* dürfte die Ursache sein, warum solche Säuren, wenn sie in grosser Quantität gegeben werden, leicht ausgeschieden werden, ohne einen Abbau erfahren zu haben, während der Organismus anscheinend die meisten von ihnen abbauen kann, wenn sie in geringer Menge zugeführt werden.

Es scheint daher *wohlmotiviert,* bei vorliegender Untersuchung auch *die d-Aminosäuren zu berücksichtigen,* auch wenn es noch nicht feststeht, dass das Nieren- und Leber-Gewebe sie unter normalen Umständen abbaut. Hierzu kommt, dass die *d- resp. l-Aminosäuren,* soweit man dies nach den bis jetzt durchgeführten Untersuchungen beurteilen kann, offenbar *durch verschiedenartige Enzyme* und auf verschiedenen Wegen *abgebaut* werden. Hierdurch wird die Begründung einer Untersuchung über die Einwirkung der Benzoesäure und naheverwandter Stoffe auf den Abbau von sowohl d- als auch l-Aminosäuren nochmals unterstrichen.

Wie früher erwähnt, hat vor allem KREBS (1932, 1933 a und b, 1935 a und b, 1939) grundlegende, ausführliche Untersuchungen über den Abbau der Aminosäuren im Nieren- und Leber-Gewebe und über die dabei wirksamen *Enzyme* vorgenommen. Auch BERNHEIM u. MITARB. (1932, 1934 a und b, 1935 a und b, 1936) haben ihren Beitrag an eingehenden Untersuchungen hierüber geliefert und besonders die Oxydation verschiedener d-Aminosäuren in diesen Geweben zum Gegenstand ihrer Forschung gemacht; ihre Versuche

haben sie hauptsächlich in vitro, mittels der Warburgschen Technik, angestellt.

Nach KREBS wird der Abbau fast aller Aminosäuren, einerlei ob sie der d- oder der l-Form angehören, im Nieren- und Leber-Gewebe nach dem von NEUBAUER (1909), KNOOP (1910) und NEUBAUER u. FROMHERZ (1910/11) aufgestellten Schema durch eine oxydative Desaminierung unter Bildung der entsprechenden Ketosäure und unter Ammoniakbildung eingeleitet.

Die *Bedingungen für* den *Abbau der d- und l-Aminosäuren stimmen* jedoch *nicht* völlig *überein*. So hängt der Abbau der l-Aminosäuren davon ab, dass das Gewebe relativ unbeschädigt und seine Zellstruktur erhalten geblieben ist. Diese Säuren werden nur in Gewebeschnitten und konzentriertem Gewebebrei abgebaut, und ihr Abbau wird durch Octylalkohol und Kaliumcyanid gehemmt; all dies im Gegensatz zu den beim Abbau der d-Aminosäuren herrschenden Verhältnissen. Dieser kann auch in verdünnten Aufschlämmungen von Gewebebrei und in Gewebeextrakten vorsichgehen. Wie oben (Seite 17) erwähnt, werden die d-Aminosäuren unter solchen Umständen — im Gegensatz zu den Verhältnissen im Totalorganismus — bedeutend rascher abgebaut als die l-Aminosäuren. KREBS nimmt daher, auf Grund all dieser Verschiedenheiten, an, dass es *zwei verschiedene Enzymsysteme* gibt: eines für die Desaminierung der l-Aminosäuren und eines für die d-Aminosäuren; jedoch nicht in der Weise, dass die beiden Systeme ganz ungleich und ganz unabhängig voneinander wären, sondern so, dass die empfindlichere l-Aminosäureoxydase das vollwertige Enzymsystem darstellt, von dem dann die d-Aminosäureoxydase nur ein weniger leicht ansprechendes Fragment wäre. Auf diese Art, meint KREBS, lasse sich *das Vorhandensein eines d-aminosäureabbauenden Enzyms verstehen* trotz der Tatsache, dass d-Aminosäuren in Niere und Leber normalerweise nicht abgebaut werden. Sonst, sagt KREBS, müsse man sich vorstellen, die Anwesenheit der d-Aminosäureoxydase sei dadurch begründet, dass die Aminosäurensynthese in den genannten Organen racemisch geschehe und zunächst Aminosäuren sowohl von d- als auch von l-Form ergebe. Die Aufgabe der d-Aminosäureoxydase würde dann sein, die d-Komponente abzubauen, sodass nur die l-Form in die Gewebe eingebaut werde. Diese Erklärung, die auch von BERNHEIM u. BERNHEIM (1935 b) ins Feld geführt worden ist, wurde von verschiedenen Seiten gestützt, unter anderm von

RODNEY u. GARNER (1938), von KNOOP (1942) und von ABDERHALDEN (1942). Indessen ist sie immer noch eine blosse Theorie, denn es scheint nicht gelungen zu sein, wirklich zu zeigen, dass die Aminosäuresynthese im Organismus racemisch erfolgt.

WARBURG u. CHRISTIAN (1938) ist es geglückt, aus dem Extrakt von Nieren- und Lebergewebe das *d-Aminosäureoxydaseenzym* ziemlich *rein* herzustellen sowie zu erkennen, wie es gebaut ist. Es besteht aus einem Proteinteil und einem Coferment, das ein Alloxazin-adenindinucleotid ist. Es ist *zu den Oxhydrasen zu rechnen* (OPPENHEIMER, 1939). Nach WARBURG u. CHRISTIAN ist es wahrscheinlich, dass das isolierte Enzym allen d-Aminosäuren gemeinsam ist. Hierüber sind jedoch die Ansichten geteilt. Zum Beispiel heben KARRER u. FRANK (1940) hervor, durch die reine d-Aminosäureoxydase würden nur gewisse d-Aminosäuren abgebaut, andere jedoch nicht. Dies bestreiten aber HOLTZ u. BÜCHSEL (1941/42), die keine Stütze für die Behauptung finden können, dass verschiedene d-Aminosäuren durch verschiedene Enzyme abgebaut würden.

Das Wahrscheinlichste dürfte sein, dass die *d-Aminosäuren* in der Regel *im Nieren- und Leber-Gewebe vom gleichen Enzym abgebaut* werden und unter oxydativer Desaminierung die entsprechende *Ketosäure und Ammoniak* nach dem gleichen Schema *bilden*.

Nach der ursprünglichen Auffassung von KREBS (1935 a) würde der *Abbau der l-Aminosäuren im Prinzip dem gleichen Schema folgen wie der der d-Aminosäuren*. Dies erscheint jedoch *nicht sehr wahrscheinlich*, wenn man nach den Ergebnissen einer grossen Zahl von später gemachten Untersuchungen urteilt. Es war nämlich nicht möglich, für die *l*-Aminosäuren ein gemeinsames Enzym oder einen gemeinsamen Weg des Abbaus nachzuweisen, der für die einzelnen Individuen ebenso generell passen würde wie das Entsprechende für die d-Komponenten. Die Verhältnisse scheinen verwickelter zu sein, und anscheinend ist es auch wirklich gelungen, nachzuweisen, dass *spezielle Abbauwege für eine grosse Anzahl von l-Aminosäuren* vorliegen. Es wäre jedoch denkbar, dass die *Desaminierung der l-Aminosäuren durch* die von v. EULER, ADLER, GÜNTHER u. DAS (1938) nachgewiesene *l(+)Glutaminsäuredehydrase* kombiniert mit der von BRAUNSTEIN u. KRITZMANN (1937/38) und BRAUNSTEIN (1939) nachgewiesenen *Glutamico-Aminopherase* auf einem allgemeineren Weg erfolgen könnte. v. EULER u. MITARB. gelang es, vor allem in der Leber, aber auch in der Niere und anderen Geweben, das Vorkom-

men einer auf l(+)Glutaminsäure spezifisch eingestellten Apodehydrase nachzuweisen. Diese Apodehydrase in Verbindung mit Codehydrase I oder II dehydriert l(+)Glutaminsäure zu Iminoglutarsäure, die durch Hydrolyse spontan in α-Ketoglutarsäure und Ammoniak zerfällt. Die Reaktion ist reversibel. BRAUNSTEIN u. KRITZMANN wiesen in der Muskulatur, aber auch in der Niere, der Leber und anderen Geweben das Vorkommen zweier spezifischer Enzyme nach, die durch sogenannte Umaminierung die Aminogruppe einer der beiden Aminodicarbonsäuren, entweder der Asparaginsäure oder der Glutaminsäure, in eine α-Ketosäure überführten, sodass entweder Oxalessigsäure oder α-Ketoglutarsäure nebst der der α-Ketosäure entsprechenden Aminosäure gebildet wurde. Auch diese Reaktionen sind reversibel, und nur l-Aminosäuren scheinen an ihnen teilnehmen zu können. Mittels einer Kombination dieser Enzyme haben BRAUNSTEIN u. BYCHKOV (1939) ein zellfreies, l-aminosäuredesaminierendes Enzymsystem hergestellt. Sie konnten nämlich mit l(+)Alanin und α-Ketoglutarsäure als Substrat und mit Pyocyanin als Wasserstoffüberträger das Alanin gemäss folgendem Reaktionsverlauf desaminieren:

1.) l(+)Alanin + α-Ketoglutarsäure ⟶ l(+)Glutaminsäure + Brenztraubensäure (Umaminierung).
2.) l(+)Glutaminsäure ⟶ α-Ketoglutarsäure + Ammoniak (Glutaminsäuredehydrierung).

Inwieweit dieser von BRAUNSTEIN u. BYCHKOV nachgewiesene Weg des Abbaus von l-Aminosäuren weiterreichende Bedeutung hat, steht unter Diskussion. COHEN u. HEKHUIS (1941) sind der Ansicht, dass es für eine solche Annahme keine Stütze gebe.

Ammoniakbildung aus Aminosäureamiden.

Im vorhergehenden wurde die Rolle der Aminosäuren als Ammoniakerzeuger behandelt. Hierbei entstammt das Ammoniak der Aminogruppe. Handelt es sich um die Amide der Aminosäuren, *Asparagin* und *Glutamin*, kann jedoch das entstehende *Ammoniak auch der Amidgruppe entstammen*, ein Prozess, der durch spezifische Enzyme, Asparaginase und Glutaminase, vermittelt wird. Die *Asparaginase*, die in der Hefe eingehend studiert worden ist (GEDDES u. HUNTER 1928, GRASSMANN u. MAYR 1933), wurde unter anderem auch im Nieren- und Leber-Gewebe verschiedener höherer Tiere

nachgewiesen (LANG 1904, v. FÜRTH u. FRIEDMANN 1910, GEDDES u. HUNTER 1928, KREBS 1935 b, SUZUKI 1936). Die *Glutaminase* wurde von KREBS (1935 b) zusammen mit der Asparaginase besonders im Nieren- und Leber-Gewebe von Kaninchen und Meerschweinchen studiert, und der Verfasser bringt gewichtige Gründe vor für die Annahme, dass es sich hierbei um zwei verschiedene Enzyme handle. Er weist auch nach, dass die Glutaminase im Nieren- und die im Leber-Gewebe offenbar nicht dem gleichen Typ angehören, und diskutiert die Möglichkeit, dass Nierenglutaminase unter physiologischen Verhältnissen nach synthetischer statt nach hydrolytischer Richtung hin arbeite, weshalb sie unter normalen Verhältnissen keine Ammoniakbildung vermittle.

Da jedoch *Glutamin* und *Asparagin* im Organismus *ammoniakbildend* auftreten, scheint es wohlmotiviert, auch den Einfluss der Benzoesäure auf die Desamidierung dieser beiden Stoffe zu untersuchen, umsomehr als es interessant ist, die Wirkung der Benzoesäure auf diese Enzyme mit derjenigen zu vergleichen, die sie auf die aminosäuredesaminierenden Enzyme ausübt.

Ammoniakbildung aus andern Amiden.

Es ist von Interesse, auch einige *einfache Amide* zu untersuchen, da auch diese *im Organismus umgesetzt* werden können. Schon v. NENCKI (1873) und SALKOWSKI (1877/78) zeigten, dass Benzamid im tierischen Organismus in Benzoesäure umgewandelt wird, und COHN (1890) wies nach, dass im Hundeorganismus Benzamid aus Benzaldehyd synthetisiert wird. In neuerer Zeit haben WAELSCH u. BUSZTIN (1937) im Nieren- und Leber-Gewebe des Pferdes eine *Benzamidase* nachgewiesen, die Benzamid aus Benzoesäure synthetisiert, im Nierengewebe aber Benzamid auch abbaut. Ferner hat BERNHARD (1938) einen Abbau verschiedener Alkylderivate des Benzamids bei Fütterungsversuchen an Hunden nachgewiesen. Aus den vorliegenden Untersuchungen (Seite 60) ergibt sich auch, dass im Lebergewebe von Ratten besonders stark das Benzamid, aber auch Acetamid und Propionamid der Desamidierung unterliegen. Aus diesem Grunde wurde auch die Wirkung untersucht, die die Benzoesäure auf die hierbei wirksamen Enzyme ausübt, obwohl die *Frage nach der Rolle dieser Amide als Ammoniakspender* unter natürlichen Verhältnissen noch *nicht* eingehender *beantwortet* zu sein scheint.

Ammoniakbildung aus der Imidazolgruppe des Histidins.

Im Histidin kommt Stickstoff in der *Aminogruppe* und im *Imidazolring* gebunden vor. Eine Ammoniakbildung kann man sich also als von diesen beiden Stellen ausgehend denken. Durch die Wirkung der *Histidase*, eines Enzyms, das bei einer Anzahl höherer Tiere in der Leber nachgewiesen wurde, wird nämlich der *Imidazolring des l(—)Histidins* geöffnet, und es wird Stickstoff in der Form von Ammoniak frei (EDLBACHER u. MITARB. 1926, 1930, 1931, 1934). Histidin kann jedoch auch durch *Desaminierung* abgebaut werden, wobei Urocaninsäure entsteht (KOTAKE u. KONISHI 1922). Der ersterwähnte Weg dürfte jedoch beim Abbau des Histidins in der Leber der wichtigere sein (EDLBACHER u. HEITZ 1942).

Da also die Histidase Ammoniakbildung im Organismus ermöglicht, und ihrer Wirkung nach ein Enzym von ganz anderer Art ist als die früher besprochenen, ist es dem Vergleich zuliebe von Interesse, auch die Wirkung zu untersuchen, die die Benzoesäure auf die Histidase ausübt.

Ammoniakbildung aus Muskeladenylsäure und nahestehenden Verbindungen.

Unter den *Purinverbindungen*, die desaminiert werden können und so als Ammoniakspender dienen können, waren besonders das *Adenosin* und die *Adenylsäure* Gegenstand der Untersuchung. So wiesen EMBDEN u. SCHUMACHER (1930) nach, dass Muskeladenylsäure im Nierengewebe Ammoniak abgibt. Ihre *Rolle als Ammoniakspender* muss jedoch *der der Aminosäuren* quantitativ *unterlegen* sein (KREBS 1933 a).

Die bei der Desaminierung von Adenosin und Adenylsäure wirksamen Enzyme wurden unter anderen von SCHMIDT (1928) in der Kaninchenmuskulatur studiert. SCHMIDT wies dabei nach, dass bei der Desaminierung der beiden Stoffe verschiedene Enzyme wirksam waren. Er zeigte auch, dass *durch Desaminierung von Muskeladenylsäure Inosinsäure und Ammoniak entstanden*. CONWAY u. COOKE (1937, 1938, 1939) haben die beiden Enzymsysteme in verschiedenen Kaninchenorganen eingehend studiert und gefunden, dass *Adenosindesaminase* besonders reichlich in den Därmen und in der Milz vorkommt, während Niere und Leber eine schwache Enzymkonzentra-

tion aufweisen. *Adenylsäuredesaminase* kommt besonders in der Skelettmuskulatur vor, in anderen Organen dagegen in beträchtlich geringerer Menge. In der Leber z. B. ist ihr Vorkommen sehr beschränkt und auch in der Niere recht unbedeutend, obschon nicht in gleichem Grade wie in der Leber.

In vorliegender Untersuchung wird auch die Einwirkung der Benzoesäure auf das adenylsäuredesaminierende Enzym studiert werden.

Apparatur und Technik.

Die Versuche wurden unter Anwendung der Warburgschen Technik (WARBURG 1926) an *Nieren- und Leber-Gewebe von Ratten* ausgeführt, wobei teils *Gewebeschnitte*, teils *Gewebeextrakt* verwendet wurden. Weiterhin wurden auch *Colibakterien* untersucht. Überall wurde der *Sauerstoffverbrauch* und die *Ammoniakbildung* bestimmt und die Wirkung verschiedener Stoffe auf diese Vorgänge studiert.

Das bei den Versuchen angewandte Verfahren soll näher geschildert werden.

Versuche mit Nieren- und Leber-Gewebe.

Bestimmung des Sauerstoffverbrauchs.

Gemeinsame Technik bei Schnitt- und Extrakt-Versuchen.

Die Versuche wurden mit Hilfe von *Warburgschen Respirationsgefässen* von ca. 16 ml Rauminhalt und mit Luft als Sauerstoffquelle ausgeführt. Die Flüssigkeitsmenge in den Gefässen und das Versuchsgewebe nahmen zusammen den Raum von 3,00 ml ein. Zwecks Absorption der Kohlensäure wurden die Einsätze mit 0,20 ml 2n-Natronlauge versehen. Wurden weitere Stoffe zugesetzt, so erfolgte dies von dem Annex aus, der sich seitlich am Gefäss befindet. Das Wasserbad hatte die Temperatur von 37,°5 C. Um vor Beginn der Versuche ein Temperatur- und Gas-Gleichgewicht herzustellen, wurden die Gefässe zuerst 15 min geschüttelt, ehe die Hähne geschlossen wurden und die Ablesung begann. Das Schütteln erfolgte mit einer Frequenz von ca. 120 Schlägen in der Minute, bei einer Amplitude von ± 2 cm.

Versuchslösungen. Als flüssiges Medium wurde Krebssche »*Phosphatsalzlösung*» (KREBS 1933 a) verwendet; diese besteht aus in Phosphatpuffer mit pH 7,4 gelöstem Natrium-, Kalium- und Calcium-Chlorid und Magnesiumsulfat. Verschiedene Enzymsubstrate, Gifte und sonstige Stoffe, die bei den Versuchen verwendet wurden, wurden in neutralisierter Lösung, im allgemeinen als *Natriumsalze* zugesetzt. Die Stoffe lagen meist als Pro-analysi-Präparate vor oder wiesen doch einen ebensohohen Reinheitsgrad auf wie solche. Manchmal wurden schwer zu beschaffende Stoffe hier hergestellt. In solchen Fällen wurde der Reinheitsgrad durch unter anderem Schmelzpunktsbestimmungen kontrolliert. Bei der Darstellung und Besprechung der

verschiedenen Versuche wird Ursprung und Reinheitsgrad der verwendeten Stoffe jeweils angegeben.

Gewebe. Die Organe wurden von *erwachsenen weissen Ratten* genommen. Die Tiere stammten sämtliche von einigen wenigen Individuen ab, und da wiederholt Inzucht erfolgt war, lag ein relativ homogenes Tiermaterial vor. — Es ist wichtig, dass die zum Versuch zu verwendenden Tiere erwachsen sind, denn es hat sich gezeigt, dass der Stoffwechsel bei nicht vollerwachsenen Tieren sowohl noch stärker variiert als auch geringer ist als bei den erwachsenen. Die gleiche Beobachtung machte WESTPHAL (1943) hinsichtlich der Wirkung von d-Aminosäureoxydase in der Rattenleber. Es wurden daher für die vorliegende Arbeit ziemlich gleichaltrige, jugendlich-erwachsene Tiere (3—6 mon alt) verwendet. Die Ernährung der Tiere war mit Milch und Küchenabfall erfolgt; die Nahrung war also vielseitig, dabei aber ziemlich homogen.

Spezielle Technik der Schnittversuche.

Bei der *Herstellung von Gewebeschnitten* ist es praktisch, sofern es sich um Leberschnitte handelt, unter den erwachsenen Tieren die jüngeren, wie bereits gesagt, nicht über 6 mon alten Individuen zu wählen. Bei ihnen ist das Bindegewebe der Leber noch nicht so fest, dass der Konsistenzunterschied zwischen diesem und dem Parenchymgewebe zu gross wird, um noch die Gewinnung gut brauchbarer Schnitte zu ermöglichen, von Schnitten nämlich, die eine genügend gleichmässige Dicke aufweisen und die nicht allzusehr zerfetzt sind. Bei Nierenschnitten kommt es darauf nicht so sehr an, da die Niere eine festere und homogenere Konsistenz hat.

Die Tiere werden durch einen Schlag in den Nacken mit darauffolgender Dekapitation getötet, wobei die Organe rasch den grössten Teil ihres Blutes verlieren. Danach werden die Organe aus dem Körper genommen und die Nieren von den Bindegewebshüllen befreit.

Die *Nieren* werden durch einen Schnitt von der Konvexität nach der Konkavität hin halbiert und das Calyxgewebe wird weggeschnitten. Die Nierenhälften werden dann fixiert, indem man sie mit der Schnittfläche nach unten gegen eine Siebplatte saugt[1]), worauf die Herstellung der Schnitte mittels des Rasiermessers sofort beginnt. Die Nierenhälften, die noch nicht gebraucht werden, werden bis auf weiteres in einer eisgekühlten Schale aufbewahrt. Soweit irgend möglich, werden die Schnitte von der Rindensubstanz genommen, und sie werden so dünn und gleichmässig hergestellt, wie es überhaupt geschehen kann. Von Lebergewebe homogene Schnitte zu erhalten, ist bedeutend schwieriger. Die *Leber* wird am besten in nierengrosse Stücke zerschnitten, von denen dann jedes für sich zur Herstellung von Schnitten dient. Zur Herstellung geeigneter Schnitte bewähren sich am besten die der Oberfläche nahen Gewebeschichten, besonders die auf der konvexen Seite gelegenen. Hier ist das Gewebe am homogensten, nicht durchsetzt mit gröberem Bindegewebe oder durchzogen von gröberen Gefässbündeln. Die Leberschnitte fal-

[1]) Eine porzellanene Siebplatte von ca. 20 mm Diameter ist in dem weiteren Ende einer schmal zulaufenden Glasröhre festgekittet. Das andere Ende der Röhre steht in Verbindung mit einer Saugpumpe.

len indessen doch immer bedeutend dicker aus als Nierenschnitte und variieren auch mehr im Gewicht. Die bereits fertigen Schnitte werden bis zur Fertigstellung der übrigen in eisgekühlter Phosphatsalzlösung aufbewahrt.

Die Schnitte werden mit der BANGschen *Torsionswage* ausgewogen, nachdem sie zuvor durch rasches Abtrocknen auf Filtrierpapier von freier Flüssigkeit befreit wurden. Bei Routineversuchen wurde auf eine nachträgliche Trockengewichtsbestimmung verzichtet.

Die *Genauigkeit dieses Verfahrens* ergibt sich aus folgenden Gewichtsversuchen. Von den Nieren eines Tieres wurden Schnitte hergestellt und hiervon 7 Portionen von je 70 mg ausgewogen. Die Gewebeportionen wurden unmittelbar nach der Wägung getrocknet, worauf eine Trockengewichtsbestimmung vorgenommen wurde. Das Trockengewicht betrug im Mittel 12,7 mg; $\sigma = \pm 0{,}33$, d. h. $\pm$ 3 %. Ein entsprechender Versuch mit 200 mg Lebergewebe ergab bei jeder einzelnen von 6 Portionen einen durchschnittlichen Trockengewichtswert von 42,5 mg; $\sigma = \pm 2{,}3$, d. h. $\pm$ 5 %. Für Lebergewebe sind also die Versuchsfehler grösser, was darauf beruhen dürfte, dass die Leberschnitte weniger homogen ausfallen und keine so glatte Schnittfläche haben, wodurch die Abtrocknung vor der Wägung nicht so gleichmässig erfolgt wie bei Nierenschnitten.

Eine *nachträgliche Trockengewichtsbestimmung* ist nach der Literatur die allgemein übliche Methode zur Bestimmung der Gewebemengen bei Schnittversuchen. Diese Methode ist aber mit einem erheblichen Fehler behaftet. Besonders bei Leberschnitten wird nämlich ein grosser Teil des Gewebes im Laufe des Versuches zu Brei, und dieser kommt dann nie vollständig mit in die Trockengewichtsbestimmung. Diese Fehlerquelle wird umgangen, wenn man sich des hier beschriebenen Verfahrens bedient, das, wie Kontrollversuche zeigten, eine gute Übereinstimmung zwischen den Gewebemengen in den verschiedenen Versuchsgefässen ergibt.

Bei der Wahl der für jedes Versuchsgefäss zu verwendenden *Schnittmenge* muss darauf Rücksicht genommen werden, dass sie so gross ist, dass der Stoffwechsel mit genügender Genauigkeit bestimmt werden kann, sowie auch darauf, dass die Schnitte von Leber und Nieren eines und desselben Tieres für eine genügende Anzahl von Einzelversuchen in je einem Warburggefäss ausreichen. Es zeigt sich nämlich, dass, wie bereits Seite 25 angedeutet, der Stoffwechsel in den Organen verschiedener Tiere auch dann recht beträchtlich variiert, wenn diese schon erwachsen sind; man muss deshalb einen vollständigen Versuch, bestehend aus 6—8 Einzelversuchen, an den Organen eines und desselben Tieres machen können. In gewissen Fällen, wenn grössere Versuche nötig waren, wurden auch Schnitte von den Organen mehrerer Tiere zusammen verwendet, aber dann variierte der Stoffwechsel in den Parallelversuchen mehr als gewöhnlich.

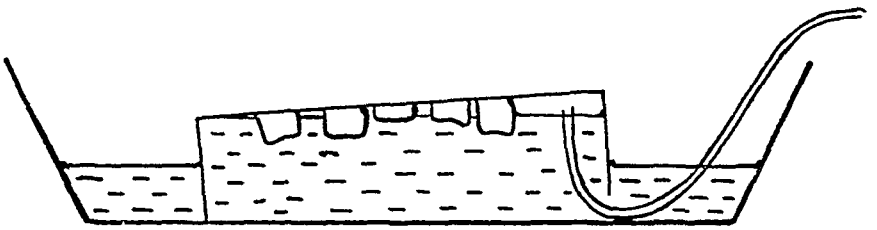
Das Endergebnis des Stoffwechsels hängt natürlich ab von der Dauer der *Versuchszeit.* Diese muss jedoch begrenzt werden, sollen nicht unerwünschte Faktoren mithereinspielen. Als geeignete Versuchsdauer wurde die *Zeit von 1½ std* betrachtet, wobei die *Ausgleichszeit* (¼ *std*) *nicht miteingerechnet* ist. In dieser Zeit dürfte weder eine Bakterienwirkung auftreten noch das Substrat aufgebraucht sein oder eine Enzymdestruktion sich in nennenswertem

Grade geltend machen, was daraus hervorzugehen scheint, dass der Sauerstoffverbrauch und die Ammoniakbildung während der ganzen Versuchszeit ungefähr gleichbleiben (Diagramme 1—8).

Mit Rücksicht auf die genannten Umstände schienen *60 mg Nierengewebe* (*Nassgewicht*) in jedem Gefäss eine geeignete Schnittmenge zu sein; entsprechend wurde für *Lebergewebe* eine Menge von *200 mg* gewählt, da hier der Stoffwechsel per Gewichtseinheit erheblich geringer ist.

Spezielle Technik der Extraktversuche.

Die Organe werden auf die oben beschriebene Weise entnommen, worauf das *Extrakt* im grossen und ganzen *gemäss* BERNHEIM u. BERNHEIM (1934 a) bereitet wird.



Figur 1. Einige Eisstücke werden unter eine umgekehrte Petrischale gelegt, die in einer zum Teil mit Wasser gefüllten grösseren Schale steht. Die Luft unter der Petrischale wird durch einen zu diesem Zwecke unter sie hineingesteckten Gummischlauch abgesaugt, sodass die Eisstücke sich unmittelbar gegen die Petrischale legen.

Die Organe werden auf eisgekühlter Unterlage (Fig. 1.) zu einem feinen Brei zerschnitten, der dann einige Minuten lang zusammen mit feingemahlenem Glas in einem Mörser zerrieben wird. Bei Nierengewebe wird hierbei die doppelte Menge Phosphatsalzlösung, bei Lebergewebe nur die gleiche Menge von solcher Lösung zugesetzt, da, wie erwähnt, die Enzymaktivität im Lebergewebe geringer ist. Darnach wird die Aufschlämmung durch eine doppelte Lage Gaze filtriert; das Filtrat stellt dann das Extrakt dar, das nun ohne weiteres zu den Versuchen verwendet werden kann. In der Regel wurde das Extrakt unmittelbar nach seiner Herstellung verwendet, da es durch Stehenlassen an Aktivität verliert. Wie die Schnitte, wurde auch das Extrakt sowohl vor wie nach der Verteilung in die Respirationsgefässe bis zum Beginn der Versuche kühl aufbewahrt.

Zur Bereitung von Nierenextrakt werden in der Regel 4 Nieren und 10 ml Phosphatsalzlösung genommen; hier wird die Gewebemischung trotz der Herkunft der Nieren von zwei verschiedenen Tieren derart homogen, dass nie ein aus solchem Vorgehen entstehender Nachteil beobachtet werden konnte. Zur Bereitung von Leberextrakt werden eine Leber und 10 ml Phosphatsalzlösung verwendet. Die *Extraktmenge* in jedem Respirationsgefäss betrug *1 ml*. Ferner werden, je nach der Menge anderer, im Laufe des Versuches zuzusetzender Lösungen etwas mehr oder weniger, jedoch ungefähr 2 ml Phosphatsalzlösung zugesetzt, bis die gesamte Flüssigkeitsmenge im Gefäss 3 ml ausmacht.

Auf diese Art wird ein Extrakt erhalten, das einen gewissen Spontanstoffwechsel beibehält, worin aber die Zellenstruktur so zerstört wurde, und die Enzyme so verdünnt sind, dass nur d-Aminosäuren, aber *keine l-Aminosäuren abgebaut werden.*

Auch bei den Extraktversuchen betrug die *Versuchsdauer* gewöhnlich *1½ std, die Ausgleichszeit (¼ std) nicht eingerechnet.*

Bestimmung der Ammoniakbildung.

Nach Abschluss der Respirationsversuche wird das vom Gewebe während der Versuchsdauer gebildete Ammoniak bestimmt. Die Bestimmung erfolgt mit Hilfe der *Diffusionsmethode nach* CONWAY u. BYRNE (1933), wenngleich *in* etwas *modifizierter Form.* Die von diesen Verfassern verwendeten Diffusionskammern sind nämlich nicht geräumig genug für die in Rede stehenden Versuche, da die Ammoniakbestimmung am ganzen Inhalt eines jeden Respirationsgefässes vorgenommen werden muss, also an 3 ml Reaktionsgemisch und weiteren 2 ml Spülflüssigkeit; die Kammern von CONWAY und BYRNE sind nur für höchstens 3 ml Analysenflüssigkeit berechnet. Es wurden deshalb aus je zwei Petrischalen von 60 resp. 40 mm Diameter und 12 resp. 7 mm Tiefe Diffusionskammern verfertigt. Die kleine Schale wurde mit einer dünnen Paraffinschicht in der grossen festgeschmolzen, worauf die ganze Kammer inwendig paraffiniert wurde. Solche Kammern sind geeignet für 5 ml Analysenflüssigkeit im Aussenraum, 5 ml 0,01 n-Salzsäure im Innenraum, sowie 1 ml gesättigter Natriumkarbonatlösung im Aussenraum, letztere dazu bestimmt, das Ammoniak freizumachen. Um das während der Diffusionszeit von der Salzsäure gebundene Ammoniak zu bestimmen, werden in der Regel 4 ml Salzsäure aufgenommen und nesslerisiert (Probe + 0,2 ml Einäscherungslösung + Wasser ad 7 ml + 3 ml NESSLERsches Reagens). Die kolorimetrische Bestimmung wird im PULFRICHschen *Photometer* mit Filter S 50 unter Anwendung einer 1-cm-Küvette ausgeführt. Die dabei erhaltenen Exstinktionswerte werden nach einem Diagramm, das nach einer Probe mit bekannten Mengen von Ammoniumsulfat hergestellt wurde, in entsprechende Milligramm Ammoniak verwandelt. Bei den Standardisierungsversuchen zeigte es sich, dass eine *Diffusionszeit* von 4 std bei Zimmertemperatur für quantitativen Austausch genügt. Bei den Versuchen war jedoch die Minimaldauer 8 std, und in der Regel betrug die Diffusionszeit sogar 12—16 std.

Zuverlässigkeit der Ammoniakanalysen.

Die *Zuverlässigkeit der Methode* ergibt sich aus folgendem Versuch, bei dem die Ammoniakmenge an 10 Probequanten bestimmt wurde. Zwei Analysen galten nur Proben eines Nierenextraktes mit unbekannter Ammoniakmenge, während die übrigen Analysen ebensolchen Proben galten, die jedoch je 1 ml Ammoniumsulfatstandard mit 0,010 mg Ammoniak enthielten. Die Proben wurden nach 12 std analysiert, wobei sich zeigte, dass die beiden ersten je 0,015 mg Ammoniak enthielten, während die 8 übrigen im Mittel 0,0245 mg davon enthielten; $\sigma = \pm 0{,}0014$, d. h. $\pm$ 6 %. Der Versuch weist also eine

befriedigende Übereinstimmung zwischen den Ergebnissen der einzelnen Analysen auf.

Ferner wurden Kontrollversuche auch angestellt, um zu untersuchen, *ob alles Ammoniak*, das während der Versuche in den Respirationsgefässen gebildet wird, *von der Reaktionslösung* bei pH 7,4 auch *gebunden wird*. Bei diesen Untersuchungen musste eine Ammoniumsulfatlösung mit einer der normalerweise in einem Nierenextrakt vorkommenden ungefähr entsprechenden Ammoniakmenge dieses in den Respirationsgefässen ersetzen. Die Gefässe wurden dann im Thermostat zunächst in gewöhnlicher Weise geschüttelt und dann sukzessive zu je zweien zwecks Ammoniakbestimmung nach ¼, ¾, 1 ¼ und 1¾ std herausgenommen. Die Ammoniumsulfatstandardlösung enthielt 0,020 mg Ammoniak. Die 8 Analysen enthielten im Durchschnitt 0,019 mg Ammoniak; $\sigma = \pm 0{,}0015$, d. h. $\pm$ 8 %. Der Versuch zeigt also, dass in dem fraglichen Punkte keine Gefahr für bedenkliche Versuchsfehler vorliegt.

Versuche mit Bakterien.

Im grossen und ganzen wurden diese Versuche auf die *gleiche Art* ausgeführt *wie die Versuche mit Nieren- und Leber-Gewebe* und nur *mit* den *Modifikationen*, die durch die Tatsache bedingt waren, dass Bakterien untersucht wurden (Webster u. Bernheim, 1936; Stephenson u. Gale, 1937).

Bei den Respirationsversuchen mit Bakterien wurde als flüssiges Medium 0,05 mol *Phosphatpuffer* mit pH 7,2 statt Phosphatsalzlösung verwendet. Als Bakteriengewebe wurde *Bacterium coli* verwendet, und zwar ein reingezüchteter, pathogener Stamm, der von einem Patienten mit Colicystopyelitis herstammte. Die Bakterien wurden vor ihrer Verwendung 18 std bei 37° C auf festem Substrat (Placentabouillon mit 2,5 % Agar-Agar) gehalten und gezüchtet, wonach sie mit steriler physiologischer Kochsalzlösung vom Substrat abgespült wurden. Danach wurden sie 4 mal zentrifugiert und mit Kochsalzlösung gewaschen und schliesslich, in solcher Lösung aufgeschlämmt, im Kühlschrank aufbewahrt, was ohne Nachteil 3—4 Tage dauern durfte. Auf diese Weise wurden sogenannte *resting bacteria* erhalten, die sich bei Respirationsversuchen durch einen geringen Spontanstoffwechsel auszeichnen, aber dabei die Fähigkeit bewahrt haben, zugesetzte Substrate umzusetzen. Von der Bakterienaufschlämmung, die ca. *5—15 mg (Trockengewicht) Bakterien per ml* enthielt, wurde bei den Respirationsversuchen in jedem Warburggefäss 1 ml verwendet. Die *Versuchsdauer* betrug in der Regel *1 std, ¼ std Ausgleichszeit nicht mitgerechnet*. Im übrigen wurden die Respirationsversuche wie auch die *Ammoniakbestimmungen* auf die früher beschriebene Art ausgeführt.

Methodologische Probleme.

Ehe die Hauptversuche in Angriff genommen wurden, wurden eine Reihe vorbereitender Kontrollversuche unter den oben in Aussicht genommenen Bedingungen angestellt, wobei diese in gewissem Masse variiert wurden; auf diese Weise sollte Klarheit über die Brauchbarkeit der Standardbedingungen und die Zuverlässigkeit der Methoden gewonnen werden.

Versuche mit Nieren- und Leber-Gewebe.

Die Reproduzierbarkeit der Sauerstoff- und Ammoniak-Analysen.

Zur Beurteilung der Versuchsresultate ist es natürlich wichtig, einen Begriff von der Zuverlässigkeit der benutzten Methoden zu haben; von einer solchen darf die Rede sein, wenn die Ergebnisse von *Parallelanalysen übereinstimmen.* Zu diesem Zwecke wurden der Sauerstoffverbrauch und die Ammoniakbildung in einer Anzahl von Versuchsserien unter verschiedenen Bedingungen studiert. So wurden Versuche mit *Nieren- und Leber-Schnitten* und *-Extrakt je bei und ohne Anwesenheit von Benzoesäure* angestellt. Auf den Einfluss der Benzoesäure soll im Folgenden näher eingegangen werden: hier sei davon bloss vorausgeschickt, dass diese Säure unter gewissen Umständen sowohl den Sauerstoffverbrauch als auch die Ammoniakbildung und zwar ebensowohl im Nieren- wie im Leber-Gewebe und bei Bacterium coli beträchtlich hemmt. Die Benzoesäure wurde hier, wie auch in den meisten folgenden Versuchen in der Konzentration von 0,025 Mol/l verwendet. — Die Versuche mit Benzoesäure wurden deshalb gemacht, weil es von Interesse war, von der Grösse der Versuchsfehler auf dem niedrigeren Stoffwechselniveau, das bei Anwesenheit dieses Stoffes vorliegt, ebenfalls eine Auffassung zu bekommen.

Aus Tabelle 1 gehen die Variationen zwischen den einzelnen Analysen unter verschiedenen Versuchsbedingungen hervor. Die Versuchszeit betrug 1¾ std.

TABELLE 1.

Gewebe	Anzahl Analysen	Sauerstoffverbrauch in mm³ Mittelwert	Ammoniakbildung in mg Mittelwert
Nierenschnitt	9	291; $\sigma = \pm 9$ d. h. ± 3 %	0,022; $\sigma = \pm 0{,}0014$ d. h. ± 6 %
Nierenschnitt + Benzoesäure ..	9	74; $\sigma = \pm 2$ » ± 3 %	0,009; $\sigma = \pm 0{,}0013$ » ± 14 %
Leberschnitt ...	7	206; $\sigma = \pm 10$ » ± 5 %	0,025; $\sigma = \pm 0{,}0018$ » ± 7 %
Leberschnitt + Benzoesäure...	8	81; $\sigma = \pm 7$ » ± 9 %	0,018; $\sigma = \pm 0{,}0009$ » ± 5 %
Nierenextrakt ...	7	71; $\sigma = \pm 2$ » ± 3 %	0,014; $\sigma = \pm 0{,}0010$ » ± 7 %
Nierenextrakt + Benzoesäure...	7	51; $\sigma = \pm 3$ » ± 6 %	0,014; $\sigma = \pm 0{,}0020$ » ± 14 %
Leberextrakt	8	121; $\sigma = \pm 4$ » ± 3 %	0,030; $\sigma = \pm 0{,}0017$ » ± 6 %
Leberextrakt + Benzoesäure...	8	103; $\sigma = \pm 2$ » ± 2 %	0,020; $\sigma = \pm 0{,}0015$ » ± 8 %

Aus der Tabelle ergibt sich, dass die *Bestimmung des Sauerstoffverbrauches* bei den Versuchen mit *Nierenschnitten sicherer* ist *als* bei denen mit *Leberschnitten*. Bei den *Extraktversuchen* sind die *Abweichungen klein*, wenn die absoluten Zahlen nicht allzuklein werden. Bei den *Ammoniakuntersuchungen* ist *die Streuung durchgehends grösser*, besonders gross natürlich, wenn die absoluten Zahlen klein sind. Die erwähnten Versuche sind nur Beispiele, die die Grössenordnung der in den folgenden Versuchen zu erwartenden Abweichungen zeigen sollen. Sind die Abweichungen im Ernstfall erheblich grösser, ist der betreffende Versuch zu kassieren, denn dann liegt Verdacht auf eine besondere Fehlerquelle vor, wie z. B. Zusammenklumpung der Schnitte in den Respirationsgefässen, was insbesondere bei Leberschnitten etwas recht Gewöhnliches ist, oder auf Undichtheit der Apparatur oder Sprünge in der Paraffinschicht der Diffusionskammern und dergleichen mehr.

Gewebemenge und Versuchsdauer.

Oben wurde gesagt, die Benzoesäure hemme unter Umständen sowohl den Sauerstoffverbrauch als auch die Ammoniakbildung. Dies zeigt sich vor allem bei den Versuchen mit Nierenschnitten Im vorhergehenden wurden auch die Gründe dafür angegeben, dass bei diesen Versuchen für jedes Versuchsgefäss 60 mg Gewebe genommen werden. Nun ist es jedoch auch von Interesse, zu untersuchen, ob die gleiche Gewebemenge auch dann geeignet ist, wenn

es gilt, die Hemmung so deutlich wie möglich in Erscheinung treten zu lassen. Deshalb wurde die *hemmende Wirkung der Benzoesäure bei Anwendung verschiedener Mengen* (120, 60 und 30 mg) *Nierenschnitt* untersucht. Es ergab sich als Mittelwert zweier Versuche mit Doppelprobe, dass die Hemmung des Sauerstoffverbrauchs 49, 49 und 42 % und die der Ammoniakbildung 34, 49 und 56 % betrug. Es scheint also, dass *die Gewebemenge keine grössere Rolle spielt*, solange sie innerhalb der besagten Grenzen variiert. Aus den Ammoniakanalysen scheint allerdings hervorzugehen, dass bei kleinerer Schnittmenge und somit also kleinerer Enzymmenge eine Tendenz zu stärkerer Hemmung vorliege; es ist jedoch zu beachten, dass bei einer so kleinen Schnittmenge die Ammoniakbildung so gering — im vorliegenden Fall 0,008 mg — wird, dass die Versuchsfehler einen recht beträchtlichen prozentualen Wert erreichen (Tab. 1), was die Abweichung erklären kann.

Im vorhergehenden wurde eine Versuchsdauer von 1¾ std als geeignet bezeichnet. Es ist da wichtig, zu untersuchen, *ob die Hemmungswirkung der Benzoesäure mit* dem Fortschreiten der *Versuchsdauer variiert* oder ob sie dabei konstant bleibt, um so zu erfahren, ob auch von diesem Gesichtspunkt aus eine Versuchsdauer von 1¾ std geeignet sei. Es wurde also die Hemmung sowohl bei Nieren- und Leber-Schnitt-Versuchen je mit und ohne Stimulierung des Stoffwechsels durch Aminosäuren nach verschiedenlanger Versuchsdauer untersucht (Diagramme 1—8). Hierbei wurde zu den markierten Zeitpunkten der Sauerstoffverbrauch in den einzelnen Proben notiert, und zwar mit und ohne Benzoesäure, und hiernach der prozentuale Wert der Hemmung errechnet. Ferner wurden zwei Proben, eine mit und eine ohne Benzoesäure, einer Ammoniakanalyse unterworfen, und es wurde hiernach der prozentuale Wert der Hemmung auch hinsichtlich der Ammoniakbildung errechnet. Die Diagramme 1—4 führen vor Augen, dass sowohl der Sauerstoffverbrauch als auch die Ammoniakbildung, ohne Benzoesäure so gut wie bei Anwesenheit von solcher, während der Versuchsdauer eine ziemlich geradlinige Mehrung zeigen, und dass sich also die *Hemmung ziemlich konstant* hält. Dies gilt in gleicher Weise für den spontanen wie für den durch l(+)Glutaminsäure stimulierten Stoffwechsel *im Nierenschnitt*. Die Diagramme 5—8 erweisen, dass für den *Leberschnitt* hinsichtlich des Stoffwechselganges ungefähr die *gleichen Verhältnisse* gelten, wobei jedoch die Ammoniakbildung im Anfang

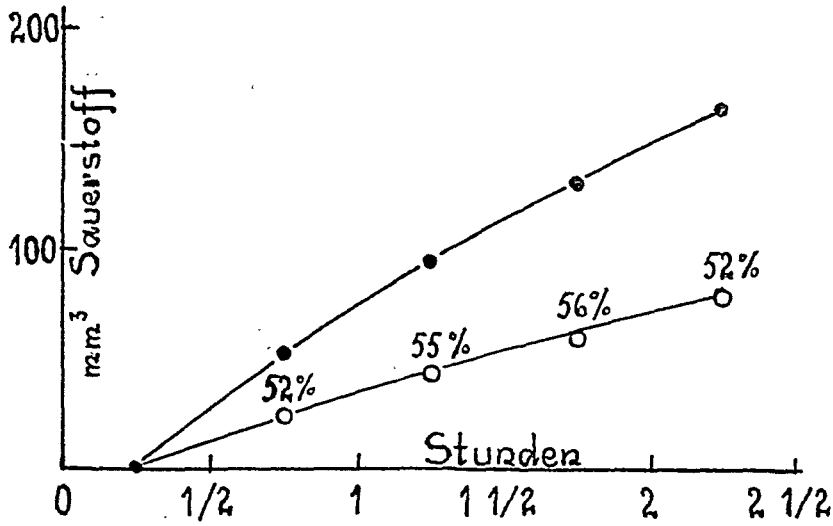


Diagramm 1. *Spontaner Sauerstoffverbrauch im Nierenschnitt* bei $2^1/_4$stündiger Versuchsdauer mit und ohne Benzoesäure in 0,025-mol Lösung. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben.

Ohne Benzoesäure . . . . . ●——●
mit » . . . . . ○——○

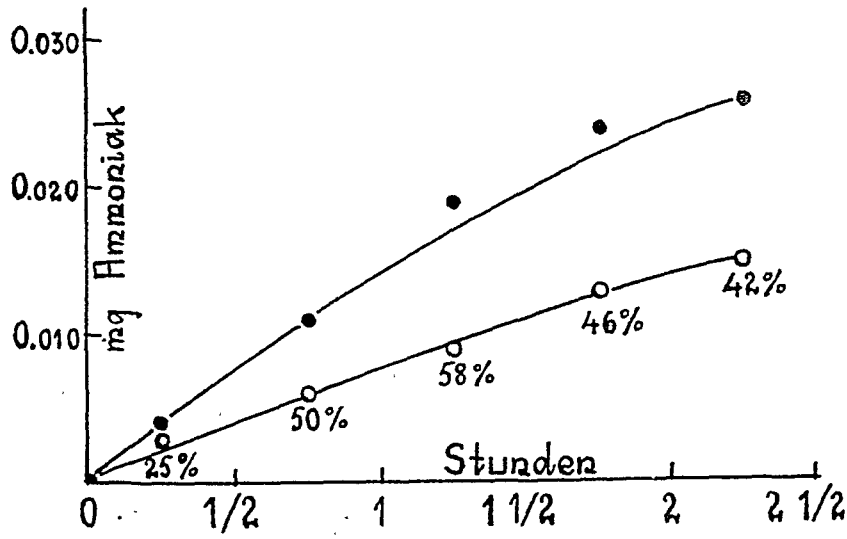


Diagramm 2. *Spontane Ammoniakbildung im Nierenschnitt.* Gleicher Versuch wie bei Diagramm 1. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben.

Ohne Benzoesäure . . . . . ●——●
mit » . . . . . ○——○

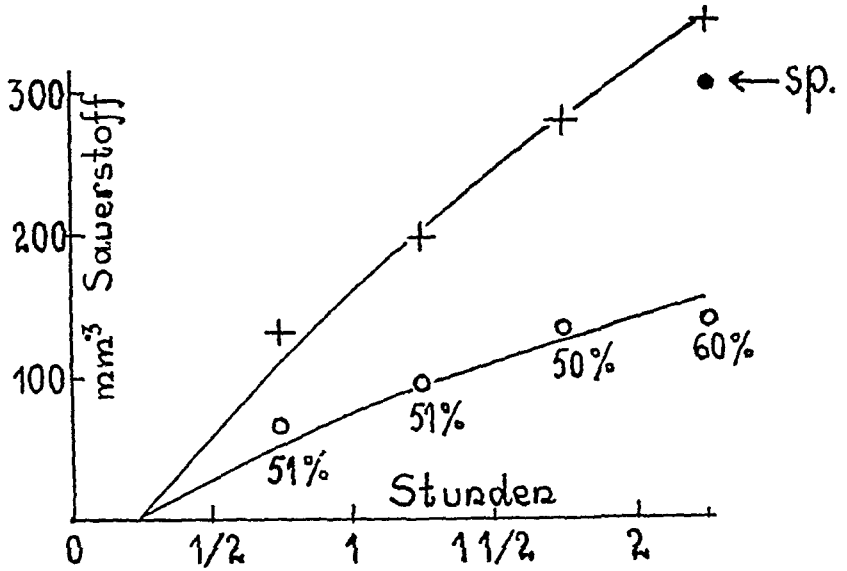


Diagramm 3. *Sauerstoffverbrauch im Nierenschnitt* bei $2^1/_4$stündiger Versuchsdauer mit *l* (+) *Glutaminsäure* von 0,0025-mol Konzentration in der Versuchslösung, mit und ohne Benzoesäure in 0,025-mol Lösung. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben. Die Grösse des spontanen Sauerstoffverbrauchs am Ende der Versuchszeit ist angegeben.

Ohne Benzoesäure . . . . . +——+
mit » . . . . . O——O

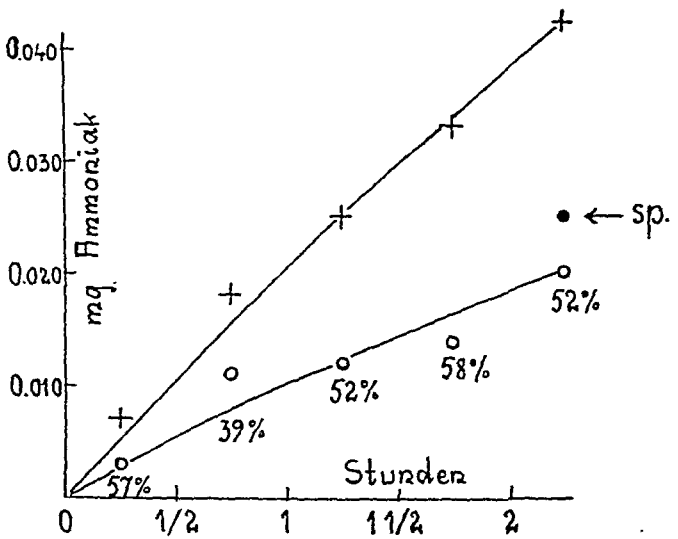


Diagramm 4. *Ammoniakbildung im Nierenschnitt.* Gleicher Versuch wie bei Diagramm 3. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben. Das Mass der spontanen Ammoniakbildung am Ende der Versuchszeit ist angegeben.

Ohne Benzoesäure . . . . . +——+
mit » . . . . . O——O

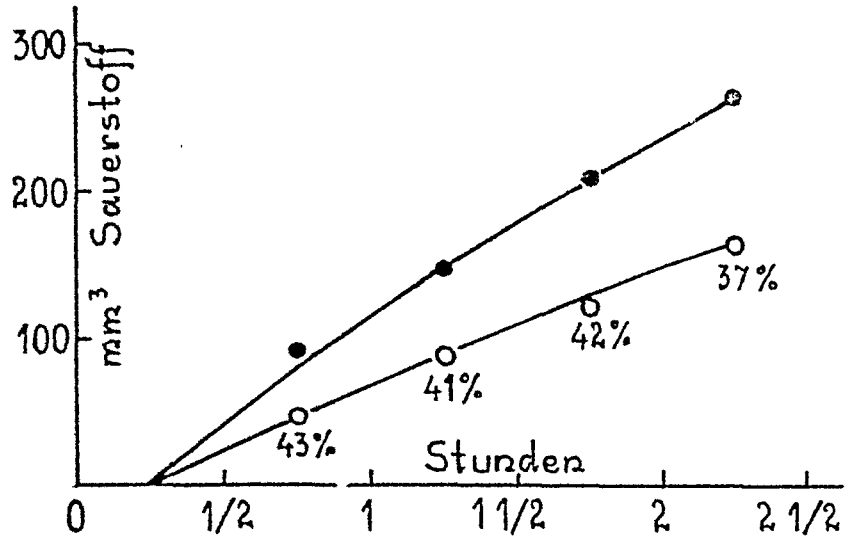


Diagramm 5. *Spontaner Sauerstoffverbrauch im Leberschnitt* bei $2^1/_4$stündiger Versuchsdauer mit und ohne Benzoesäure in 0,025-mol Lösung. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben.

Ohne Benzoesäure . . . . . ●——●
mit » . . . . . ○——○

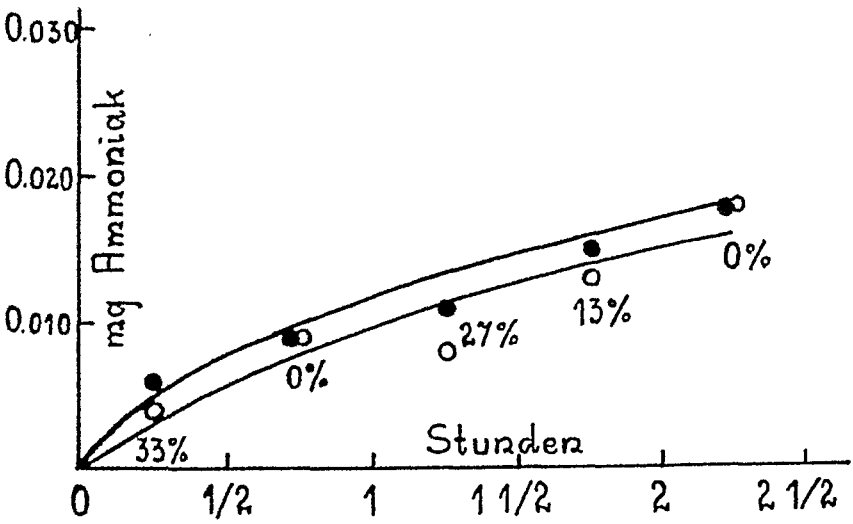


Diagramm 6. *Spontane Ammoniakbildung im Leberschnitt.* Gleicher Versuch wie bei Diagramm 5. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben.

Ohne Benzoesäure . . . . . ●——●
mit » . . . . . ○——○

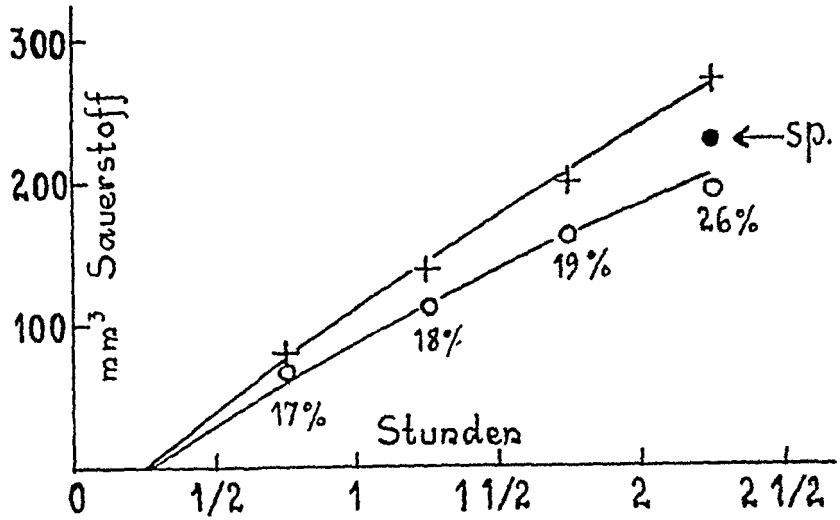


Diagramm 7. *Sauerstoffverbrauch im Leberschnitt* bei 2¼stündiger Versuchsdauer mit *l* (—) *Asparaginsäure* von 0,01-mol Konzentration in der Versuchslösung, mit und ohne Benzoesäure in 0,025-mol Lösung. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure verursachte Hemmung ist in Prozenten angegeben. Die Grösse des spontanen Sauerstoffverbrauchs am Ende der Versuchszeit ist angegeben.

Ohne Benzoesäure . . . . . +——+
mit » . . . . . O——O

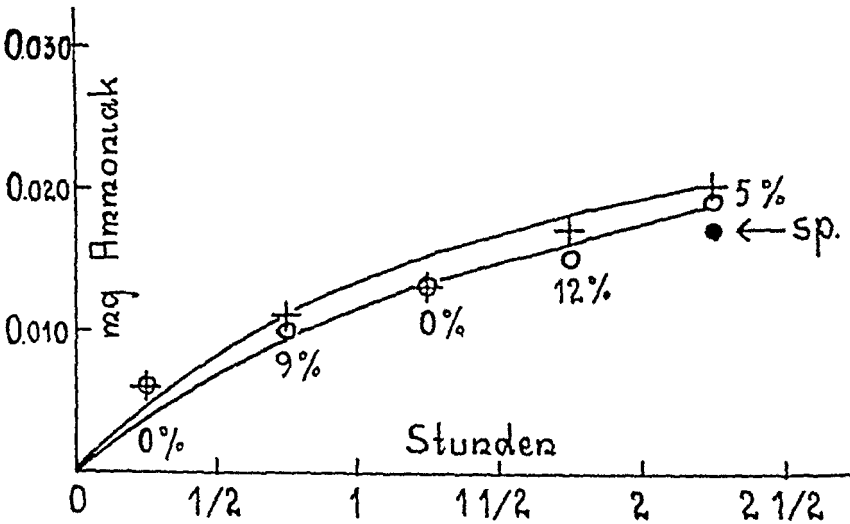


Diagramm 8. *Ammoniakbildung im Leberschnitt.* Gleicher Versuch wie bei Diagramm 7. Die zum Zeitpunkt der verschiedenen Ablesungen durch die Benzoesäure hervorgerufene Hemmung ist in Prozenten angegeben. Das Mass der spontanen Ammoniakbildung am Ende der Versuchszeit ist angegeben.

Ohne Benzoesäure . . . . . +——+
mit » . . . . . O——O

etwas rascher vor sich geht. Es liegt indessen ein wesentlicher Unterschied zwischen Nieren- und Leber-Schnitt insofern vor, als Benzoesäure im Leberschnitt eine schwächere Hemmung auf den Sauerstoffverbrauch und keine sichere Hemmung auf die Ammoniakbildung ausübt.

Im *Nieren- und Leber-Extrakt* (Diagramme 15—18) scheint sich die Hemmungswirkung anders zu äussern als in den Schnittversuchen, weshalb die Besprechung dieser Diagramme später in anderem Zusammenhang erfolgen soll.

Zusammenfassend kann man also, gestützt auf das Ergebnis der Versuche über die hemmende Wirkung der Benzoesäure bei verschiedenlanger Versuchsdauer, sagen, dass auch in dieser Hinsicht der Festsetzung der Versuchszeit auf 1¾ std nichts im Wege steht.

Die Stärke des Stoffwechsels.

Weiterhin ist es interessant, zu untersuchen, ob die *Stärke des Stoffwechsels*, wie er *unter den hier angegebenen Versuchsbedingungen* stattfindet, derjenigen entspricht, die im allgemeinen in der Literatur angegeben wird. Es ist üblich, die Grösse des Sauerstoffverbrauchs und das Mass der Ammoniakbildung in Kubikmillimetern des per Milligramm Trockengewicht des Gewebes während einer Stunde aufgenommenen Sauerstoffs oder während dieser Zeit gebildeten Ammoniaks anzugeben. Diese *Quoten* bezeichnet man mit Q_{O_2} und Q_{NH_3}. Eine Berechnung dieser Quoten für den Spontanstoffwechsel im Nierenschnitt ergibt bei einem der Versuche die Werte $Q_{O_2} = 16{,}8$ und $Q_{NH_3} = 1{,}93$ bei einem Sauerstoffverbrauch von 275 mm³ und einer Ammoniakbildung von 0,024 mg bei einer Versuchsdauer von 1¾ std und 60 mg (Nassgewicht) Nierenschnitt, was ca. 10,9 mg Trockengewicht entspricht. In drei Versuchen von Krebs (1935 a) sind die entsprechenden Werte 19,2; 21,0; 24,1 resp. 1,40; 3,28; 2,39. Bei entsprechender Berechnung der durch 0,01-mol l(+)Glutaminsäurelösung stimulierten Ammoniakbildung im Nierenschnitt, erhielt ich in einem Versuch für Q_{NH_3} den Wert 3,0. Über einige gut damit vergleichbare Versuche berichten Kögl, Herken u. Erxleben (1940), die die Q_{NH_3}-Werte für 9 Versuche als zwischen 2,4 und 7,3 variierend angeben. Hier ist jedoch zu beachten, dass die Verfasser die Aminosäure in einer Konzentration von 0,025 Mol/l verwendeten. Es scheint somit, als sei der Stoffwechsel bei den in vorliegender Arbeit abgehandelten Versuchen von

gleicher Grössenordnung wie in den Versuchen Anderer, wenn er auch im allgemeinen etwas niedriger zu liegen scheint.

Aus den oben referierten Versuchen von KÖGL u. MITARB. ergibt sich, dass der *Stoffwechsel bei verschiedenen Individuen der gleichen Tierart* recht stark *variieren kann*, und dies wurde auch hier schon erwähnt (Seite 26). Manchmal kommt *auch*, besonders bei den jüngsten der verwendeten Tiere, eine recht *beträchtliche Abweichung der Hemmungswirkung der Benzoesäure* und anderer verwendeter Stoffe auf den Stoffwechsel vor, auch wenn die Stärke des Stoffwechsels ziemlich konstant ist. Tabelle 6 und 7 zeigen die Grösse der Abweichungen bei verschiedenen Versuchen sowohl was den Stoffwechsel als was die Hemmung angeht.

Für die folgenden Versuche ist es ferner von Interesse, einen Begriff davon zu bekommen, eine *wie grosse Quantität* der bei gewissen Versuchen zwecks Stimulation der Ammoniakbildung zugesetzten *Aminosäuren während* der Dauer *der Versuche abgebaut* wird. Die Erfahrung zeigt, dass bei hoher Konzentration bloss ein geringer Teil am Stoffwechsel teilnimmt. In einem Nierenschnittversuch (Tab. 8 u. Diagr. 14) mit 0,01-mol l(+)Glutaminsäure als Stimulans stieg demgemäss zwar die Ammoniakbildung um 125 % über die spontane, wohingegen nur ca. 5 % der zugesetzten Aminosäure während der Versuchsdauer desaminiert wurden. In einem andern Versuch, mit l(+)Glutaminsäure in einer Konzentration von 0,00625 Mol/l als Stimulans, erhöhte sich die Ammoniakbildung um 40 %, und in diesem Falle wurden ca. 25 % der zugesetzten Aminosäure desaminiert.

Versuche mit Bakterien.

Die Reproduzierbarkeit der Sauerstoff- und Ammoniak-Analysen.

Es wurde der Grad der *Übereinstimmung* zwischen den Resultaten *paralleler Analysen* untersucht. In einer Serie von 9 Einzelanalysen, die unter Standardbedingungen mit 0,00125-mol dl-Alaninlösung als Substrat ausgeführt wurden, wurden 120 mm³ O_2 verbraucht; $\sigma = \pm 6$, d. h. ± 5 %. Dabei wurden 0,031 mg Ammoniak gebildet; $\sigma = \pm 0{,}001$, d. h. ± 3 %. Ein entsprechender Versuch, jedoch mit 0,1-mol Benzoesäurelösung, ergab die Werte 75 mm³; $\sigma = \pm 4$, d. h. ± 5 %, resp. 0,014 mg; $\sigma = \pm 0{,}0014$, d. h. ± 10 %. Diese Versuche sind, wie es auch bei den Versuchen mit Nieren- und Leber-

Schnitten der Fall war, nur Beispiele, die die Grössenordnung der bei den Bakterienversuchen vorliegenden Abweichungen zeigen.

Bakterienmenge und Versuchsdauer.

Um den *Einfluss der Bakterienmenge auf die Hemmungswirkung der Benzoesäure* zu studieren, wurden ebenfalls besondere Versuche angestellt. In einem Versuch mit 4,9 mg (Trockengewicht) Bakt. per ml und 0,00125-mol dl-Alaninlösung als Substrat hemmte 0,025-mol Benzoesäurelösung den Sauerstoffverbrauch um 29 % und die Ammoniakbildung um 32 %. In einem andern Versuch mit 14,1 mg (Trockengewicht) waren die entsprechenden Werte 32 % resp. 35 %. Entsprechende Versuche mit Mandelsäure und Salicylsäure ergaben eine ungefähr gleiche Übereinstimmung. Es lässt sich also *kein Unterschied in der Hemmungswirkung* nachweisen, wenn die Bakterienmenge innerhalb der hier eingehaltenen Grenzen variiert.

Im Gegensatz zu den für Gewebeschnitt-Versuche geltenden Verhältnissen bedeutete es bei den Bakterienversuchen keinerlei Schwierigkeit, ausreichend viel Gewebe von gleichem Stoffwechsel für eine grössere Anzahl von Einzelversuchen zu erhalten. Hier wurde daher sowohl die ungehemmte als auch die gehemmte Ammoniakbildung am Ende der Ausgleichszeit sowie ½ und 1 std danach registriert. Der Sauerstoffverbrauch wurde zu den zwei letztgenannten Zeitpunkten registriert. Die Stärke der Hemmungswirkung der Benzoesäure und der übrigen hierauf untersuchten Stoffe wurde nach dem Stoffwechselwert berechnet, der für das Ende der Versuchszeit galt. *Das Stärkeverhältnis des Stoffwechsels* war nämlich *während der ganzen Versuchszeit ungefähr das gleiche*, aber die grösseren absoluten Zahlen am Ende der Versuchszeit gewährleisten eine grössere Sicherheit der Berechnungen (Diagr. 21 u. 22).

Die Stärke des Stoffwechsels.

Bei der Berechnung der Aminosäurequantitäten, die während der Versuchszeit abgebaut werden, zeigen sich recht grosse Abweichungen. Im allgemeinen werden etwa 25—50 % der zugesetzten Aminosäure desaminiert.

Eigene Untersuchungen.

Die Ergebnisse der vorliegenden Untersuchungen stammen in der Regel aus je *zwei Versuchen*, bei deren jedem eine *Doppelanalyse* gemacht wurde. Nur in gewissen Fällen, nämlich wenn mehrere Untersuchungen mit ähnlichen Konzentrationen des gleichen Stoffes angestellt wurden, wurde für jede Konzentration nur e i n Versuch mit Doppelanalyse gemacht.

Der Einfluss der Benzoesäure und der Mandelsäure auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Nieren- und Leber-Gewebe.

Die Untersuchungen über die Einwirkung der Benzoesäure und der Mandelsäure auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Nieren- und Leber-Gewebe wurden *in erster Linie* mit Hilfe von *Gewebeschnitt-Versuchen* gemacht. Hier weichen die Versuchsbedingungen weniger von den natürlichen Verhältnissen ab als in Extraktversuchen, bei denen der Stoffwechsel erheblich reduziert ist. Dies geht unter anderm aus den bereits referierten Untersuchungen von Krebs (1935 a) hervor, die zeigten, dass in Extraktversuchen keine l-Aminosäuren abgebaut werden.

Der Einfluss der Stoffe in 0,025-mol Lösung auf den Stoffwechsel im Nieren- und Leber-Schnitt.

Die Versuche wurden mittels der auf Seite 24 ff. angegebenen Methode ausgeführt. Im Nierenschnitt wurden Benzoesäure und Mandelsäure in verschiedenen Konzentrationen untersucht (Seite 43), da es sich gezeigt hatte, dass beide Stoffe den Sauerstoffverbrauch und die Ammoniakbildung stark hemmen (Diagramme 9 und 10). Im Leberschnitt konnte dagegen keine sichere Einwirkung auf die Ammoniakbildung wahrgenommen werden, und die auf den Sauerstoffverbrauch ausgeübte Hemmung war schwächer als im Nierenschnitt.

Zwecks etwas näherer Beleuchtung dieser Verhältnisse soll ein Vergleich zwischen der Wirkung der beiden Stoffe bei einer Konzen-

tration von *0,025 Mol/l im Reaktionsgemisch* angestellt werden. Diese Konzentration wurde deshalb gewählt, weil bei ihr die Stoffe eine Hemmung von ca. 50 % auf die Ammoniakbildung im Nierenschnitt ausüben (Diagramm 10) und also eine unzweifelhafte, jedoch nicht ihre maximale Hemmungswirkung zeigen. Bei dieser Konzentration kann man annehmen, dass unerwünschte Begleitumstände eine kleinere Rolle spielen als bei andern Konzentrationen. Bei höherer Konzentration beginnt nämlich eine *unspezifische Salzwirkung* sich deutlich geltend zu machen.

So hebt FRANKE (1940/41) hervor, dass keine höhere Konzentration als 0,1 Mol/l für die Stoffe gewählt werden dürfe, deren Wirkung auf d-Aminosäureoxydase untersucht werden soll, da Natriumchloridlösung von dieser Konzentration die Wirkung dieses Enzyms um ca. 25 % hemme.

Eine geringere Konzentration als 0,025 Mol/l wurde ebenfalls als nicht recht geeignet betrachtet, weil dann die Hemmung nicht mehr in genügender Deutlichkeit auftritt.

Aus Tabelle 2 ergibt sich *die Hemmung, die Benzoesäure und Mandelsäure* unter oben erwähnten Versuchsbedingungen *hervorrufen*. Die Werte hier stellen die Mittelwerte sämtlicher Versuche dar (Tabelle 2 ist ein Auszug aus den Tabellen 6 und 7, in denen sich die Werte für jeden einzelnen Versuch finden lassen). Die *Wirkung von Natriumchlorid* ist ebenfalls angegeben, um die Stärke der unspezifischen Salzwirkung darzulegen.

TABELLE 2.

Die von einer 0,025 -mol Lösung von Natriumchlorid, Benzoesäure und Mandelsäure auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Nieren-und Leber-Schnitt ausgeübte Hemmung.

Stoff	Nierenschnitt Hemmung in %		Leberschnitt Hemmung in %	
	Sauerstoff-verbrauch	Ammoniak-bildung	Sauerstoff-verbrauch	Ammoniak-bildung
Natriumchlorid	10	9	4	5
Benzoesäure	66	52	48	6
Mandelsäure	39	50	28	4

Aus Tabelle 2 scheint hervorzugehen, dass Natriumchlorid auf den Sauerstoffverbrauch und die Ammoniakbildung sowohl im Nieren- als auch im Leber-Schnitt eine leichte Hemmung ausübt. Diese Hemmung könnte ihrer Stärke nach gut mit der von FRANKE angege-

benen Salzwirkung übereinstimmen. Anderseits aber sind die Werte so niedrig, dass sie auf Versuchsfehler bezogen werden können. Aus derselben Tabelle ergibt sich vor allem, dass sowohl *Benzoesäure* als auch *Mandelsäure* eine deutliche *Hemmung auf* sowohl den *Sauerstoffverbrauch* als auch die *Ammoniakbildung im Nierenschnitt* ausüben. Die von der Mandelsäure auf den Sauerstoffverbrauch ausgeübte Wirkung ist jedoch etwas geringer als die durch die Benzoesäure verursachte Hemmung, was im Folgenden näher besprochen werden soll. Im *Leberschnitt* üben die beiden Stoffe ebenfalls eine klare *Hemmung auf* den *Sauerstoffverbrauch* aus, und auch hier ist die Wirkung der Mandelsäure schwächer. Auf die *Ammoniakbildung* wirken indessen die beiden Stoffe so *wenig* ein, dass sich ihre Wirkung von der durch Natriumchlorid hervorgerufenen nicht unterscheidet. Die Stoffe üben somit nicht sicher eine Hemmung auf die Ammoniakbildung im Leberschnitt aus.

Indessen ist es denkbar, dass dieses *Fehlen einer Hemmung* der Ammoniakbildung im Leberschnitt bloss *scheinbar* vorliegt. In der Leber wird nämlich ein Teil des Ammoniaks als *Harnstoff* gebunden. Diese Harnstoffbildung geht, so nimmt man an, hauptsächlich auf dem Weg über den von KREBS u. HENSELEIT (1932) beschriebenen Ornithin-Arginin-Cyclus vor sich. Deshalb könnte man denken, dass sich die Quantität Ammoniak, die als Harnstoff gebunden wird, durch die Einwirkung der Benzoesäure und der Mandelsäure vermindere, während die nicht als Harnstoff gebundene Ammoniakmenge von diesen Stoffen nicht beeinflusst werde.

Um hier klar zu sehen, wurde bei einigen Leberschnitt-Versuchen, bei denen der Einfluss der Benzoesäure untersucht wurde, auch das Ammoniak bestimmt, das durch die Einwirkung von Urease[1]) aus dem beim Versuch gebildeten Harnstoff freiwurde. Das Ergebnis ist in Tabelle 3 dargestellt.

Aus dieser Tabelle ist ersichtlich, dass kein Unterschied bestehen dürfte zwischen der von der Benzoesäure auf diese die totale Ammoniakbildung und der auf die früher untersuchte, die Harnstoffbildung nicht mitumfassende Ammoniakbildung ausgeübten Hemmung.

Geleitet von den Werten in Tabelle 2, kann man schon ahnen, *welche ammoniakbildenden Prozesse im Nierenschnitt* es sind, die von Benzoesäure und Mandelsäure *gehemmt* werden.

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[1]) Urease sicc., Grübler & Co.

TABELLE 3.

Der Einfluss einer 0,025-mol Lösung von Benzoesäure auf den Sauerstoffverbrauch und die Ammoniakbildung im Leberschnitt. Hierein ist das Ammoniak einbezogen, das durch Harnstoffbildung gebunden wird.

Sauerstoffverbrauch in mm³		Hemmung in %	Ammoniakbildung in mg		Hemmung (—) resp. Stimulierung (+) in %
ohne Benzoesäure	mit Benzoesäure		ohne Benzoesäure	mit Benzoesäure	
233	150	36	0,040	0,034	—15
202	130	36	0,048	0,049	+2

Dass sowohl Sauerstoffverbrauch als auch Ammoniakbildung gehemmt werden, spricht dafür, dass die Oxydation von solchen Stoffen gehemmt wird, durch deren Abbau Ammoniak freiwird, d. h. der *Abbau von Aminosäuren*. Eine andere denkbare Erklärung wäre auch, dass die Stoffe ganz verschiedene Typen von Prozessen hemmten: teils Oxydationsprozesse, die keine Ammoniakbildung veranlassen, teils Prozesse, bei denen Ammoniak anaerob, zum Beispiel durch hydrolytische Desamidierung von Asparagin und Glutamin, freiwird. Diese Frage wird in den folgenden Untersuchungen ausführlicher behandelt.

Der Einfluss der Stoffe in verschiedenen Konzentrationen auf den Stoffwechsel im Nieren- und Leber-Schnitt.

Es ist von Interesse, die Einwirkung der Stoffe auch bei schwächerer Konzentration zu studieren, um zu versuchen festzustellen, *bei welcher Verdünnung* noch eine *Hemmung nachweisbar* ist. Von Bedeutung ist in erster Linie die Hemmung der Ammoniakbildung. Es zeigt sich, dass Benzoesäure und Mandelsäure nur im Nierenschnitt eine sichere Hemmung auf die Ammoniakbildung ausüben, während im Leberschnitt die Ammoniakbildung offenbar nicht beeinflusst wird. Die folgenden Untersuchungen befassen sich daher hauptsächlich mit dem Sauerstoffverbrauch und der Ammoniakbildung im Nierenschnitt.

Nierenschnitt.

Die Versuche wurden auf gleiche Weise wie die früher beschriebenen ausgeführt.

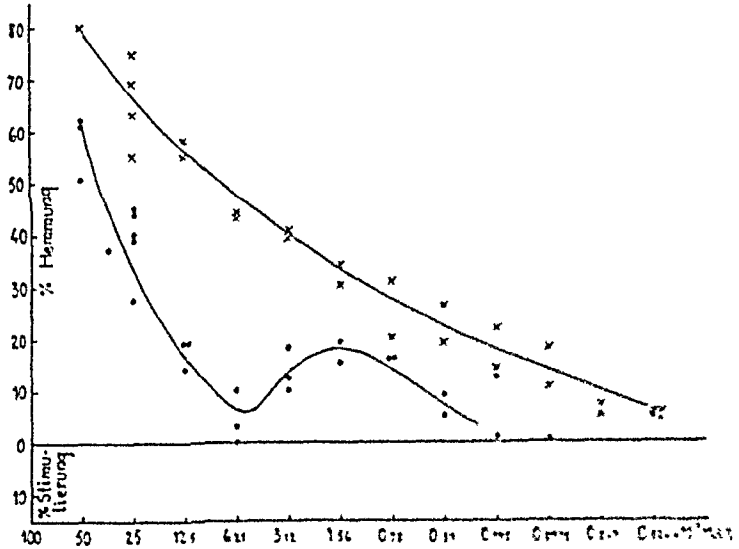


Diagramm 9. Hemmung des *spontanen Sauerstoffverbrauchs* durch Benzoesäure und Mandelsäure in verschiedenen Konzentrationen bei Versuchen mit *Nierenschnitten*. Jeder Wert gründet sich auf einen besonderen Versuch, der in der Regel mit Doppelprobe ausgeführt wurde. Die Kurven sind nach Augenmass gezogen.

Benzoesäure . . . . . ×——×
Mandelsäure . . . . . ●——●

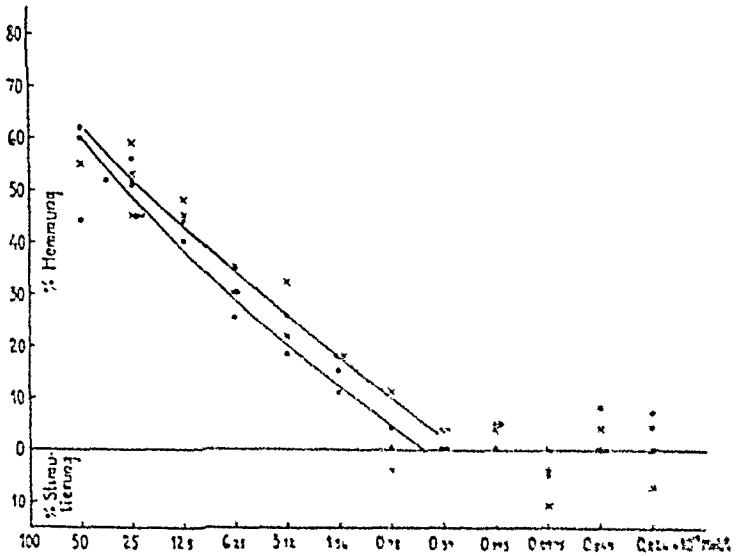


Diagramm 10. Hemmung der *spontanen Ammoniakbildung* durch Benzoesäure und Mandelsäure in verschiedenen Konzentrationen bei Versuchen mit *Nierenschnitt*. Die Werte sind den gleichen Versuchen entnommen wie die von Diagramm 9. Die Kurven sind nach Augenmass gezogen.

Benzoesäure . . . . . ×——×
Mandelsäure . . . . . ●——●

Aus Diagramm 9 und 10 geht die Stärke der *Hemmung* des Sauerstoffverbrauchs und der Ammoniakbildung im Nierenschnitt hervor, wie sie *bei verschiedener Konzentration* durch die beiden Stoffe hervorgerufen wird. Der Grad der Hemmung wurde in Prozenten angegeben. Es zeigt sich, dass die durch Benzoesäure verursachte Hemmungswirkung auf den *Sauerstoffverbrauch* bis hinunter zu einer Konzentration von ca. $0{,}0975 \times 10^{-3}$ Mol/l noch zu merken ist. Die Wirkung der Mandelsäure scheint etwas schwächer zu sein. Bei einer Konzentration von $0{,}39 \times 10^{-3}$ Mol/l, also bei einer Konzentration, die 4mal stärker ist als der für die Benzoesäure angegebene Grenzwert, kann keine sichere Hemmungswirkung mehr nachgewiesen werden.

Ein bemerkenswerter *Unterschied* besteht *hinsichtlich des Verlaufs der Kurven*, die bei der Darstellung der Werte der Hemmungswirkung entstehen, die die betreffenden Stoffe bei verschiedenen Konzentrationen auf den Sauerstoffverbrauch ausüben. Während die Kurve, die die Hemmungswirkung der Benzoesäure veranschaulicht, nur schwach bogenförmig ist und somit zeigt, dass die Wirkung proportional zum Sinken der Konzentration ständig abnimmt, zeigt die Kurve für die entsprechende Wirkung der Mandelsäure eine ausgeprägte S-Form, die erweist, dass die Kurve eine Funktion verschiedener Prozesse ist. Die Hemmungswirkung der Mandelsäure nimmt also ebenfalls sukzessive ab und zwar erreicht sie bei einer Konzentration von $6{,}25 \times 10^{-3}$ Mol/l ungefähr den Wert Null; in den nächstschwächeren Konzentrationen tritt sie jedoch wieder in Erscheinung und fällt erst bei einer Konzentration von $0{,}39 \times 10^{-3}$ Mol/l definitiv hinab auf Null.

Es besteht Anlass zu der Vermutung, der Prozess, der hier eingreift und die Kurve über die Hemmungswirkung der Mandelsäure deformiert, sei die *gleichzeitige Verbrennung der Mandelsäure*. Durch eine frühere Untersuchung wies ich (HERNER 1942) als wahrscheinlich nach, dass Mandelsäure sowohl im Nieren- als auch im Leber-Gewebe verbrannt wird. Da ist der Gedanke berechtigt, der durch die Verbrennung der Mandelsäure gemehrte Sauerstoffverbrauch werde die durch die Mandelsäure verursachte Hemmung des durch andere Oxydationsprozesse bedingten Sauerstoffverbrauchs teilweise verbergen. Dies könnte also erklären, wieso die Hemmungswirkung der Mandelsäure sowohl im Nieren- als auch im Leber-Gewebe geringer erscheinen könnte als sie ist.

Die Annahme könnte gestützt werden durch die Tatsache, dass *der hemmende Einfluss der Benzoesäure und der Mandelsäure auf die Ammoniakbildung* im Nierenschnitt bei verschiedener Konzentration dieser Stoffe untereinander *ziemlich ähnlich* sind (Diagramm 10). Auch die Versuche mit Nierenextrakt stützen diese Annahme, weshalb die Diskussion dieser Dinge im Zusammenhang mit der Besprechung der letztgenannten Versuche wieder aufgenommen werden soll.

Leberschnitt.

Die Versuche wurden auf gleiche Art wie die oben beschriebenen ausgeführt, nur wurden hier ausser den früher geschilderten Versuchen mit Benzoesäure und Mandelsäure in 0,025-mol Lösung auch Versuche mit diesen Stoffen in 0,1-mol Lösung angestellt. Die Hemmungswirkung der Stoffe geht aus Tabelle 4 hervor.

TABELLE 4.

Der hemmende Einfluss von Benzoesäure und Mandelsäure in 0,1-mol Lösung auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Leberschnitt.

Stoffe	Sauerstoffverbrauch		Ammoniakbildung	
	Hemmung in %	Mittelwert	Hemmung in %	Mittelwert
Benzoesäure	78 74	76	22 25	24
Mandelsäure	68 55	62	36 28	32

Es zeigt sich, dass die Stoffe auch in dieser Konzentration *keine besonders kräftige Wirkung auf die Ammoniakbildung* ausüben.

Es ist also nicht möglich gewesen, eine so starke Hemmung wie die in früheren Versuchen (HERNER 1942) mit Mandelsäure in 0,1-mol Lösung erhaltene zu reproduzieren. Damals war eine Hemmung von 83 % erhalten worden. Der grosse Unterschied könnte darauf beruhen, dass in den früheren Versuchen nicht beachtet worden war, dass es wichtig ist, erwachsene Tiere ungefähr gleichen Alters zu verwenden.

Der Einfluss der Stoffe in verschiedenen Konzentrationen auf den Stoffwechsel im Nieren- und Leber-Extrakt.

Nierenextrakt.

Die Versuche wurden nach der auf Seite 24 ff. angegebenen Methode ausgeführt.

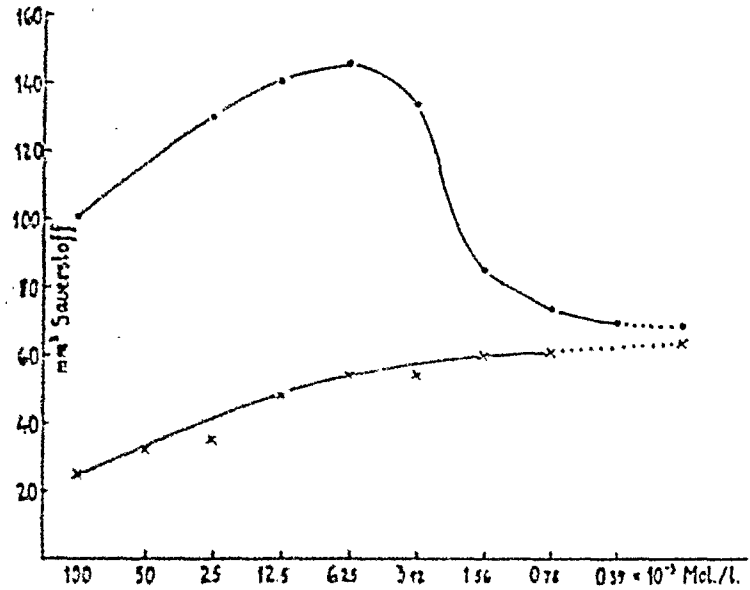


Diagramm 11. Grösse des *spontanen Sauerstoffverbrauchs* bei Versuchen mit *Nierenextrakt* ohne und bei Anwesenheit von Benzoesäure und Mandelsäure in verschiedenen Konzentrationen. Die Werte ganz rechts geben die Stärke des Stoffwechsels ohne die betr. Stoffe an.

Benzoesäure . . . . . ×——×
Mandelsäure . . . . . ●——⊙

Aus Diagramm 11 geht die Grösse des Sauerstoffverbrauchs in Nierenextraktversuchen ohne und bei Anwesenheit von Benzoesäure und Mandelsäure in verschiedenen Konzentrationen von 0,1 Mol/l—$0{,}39 \times 10^{-3}$ Mol/l hervor. Hieraus ergibt sich, dass *Benzoesäure* eine mit dem Steigen der Konzentration von ca. $6{,}25 \times 10^{-3}$ Mol/l auf 0,1 Mol/l wachsende *Hemmung* ausübt, während Mandelsäure in keiner dieser Konzentrationen eine Hemmung auszuüben scheint. Statt dessen *stimuliert Mandelsäure* von einer Konzentration von ca. $1{,}56 \times 10^{-3}$ Mol/l an bis hinauf zu 0,1 Mol/l den *Sauerstoffverbrauch* weit über den Wert des spontanen Verbrauchs hinaus. Es ist von Interesse, dass der Sauerstoffverbrauch bei einer Konzentration von ungefähr $6{,}25 \times 10^{-3}$ Mol/l stark stimuliert wird. Bei dieser und ihr naheliegenden Konzentrationen übt nämlich die Mandelsäure nach Diagramm 9 eine schwächere Hemmung auf den Sauerstoffverbrauch aus als in sowohl stärkeren als auch schwächeren Konzentrationen Dieser Umstand spricht also dafür, dass das Absinken der die Hemmungswirkung der Mandelsäure auf den Sauerstoffverbrauch im Nierenschnitt darstellenden Kurve gerade bei einer Konzentration von $6{,}25 \times 10^{-3}$ Mol/l darauf beruhe, dass die Oxydation

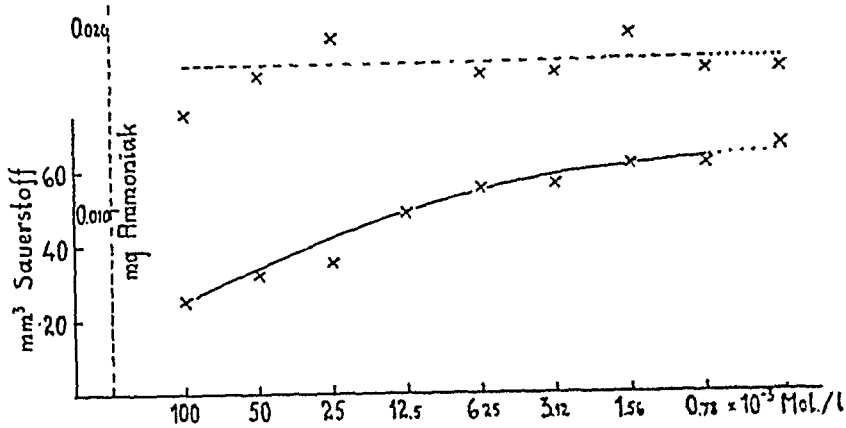


Diagramm 12. Mass des *spontanen Sauerstoffverbrauchs* und der *spontanen Ammoniakbildung* bei Versuchen mit *Nierenextrakt* ohne und bei Anwesenheit von Benzoesäure in verschiedenen Konzentrationen. Die Werte ganz rechts geben die Stärke des Stoffwechsels ohne Benzoesäure an

Sauerstoffverbrauch . . . . . ×———×
Ammoniakbildung . . . . . × - - - - - ×

der Mandelsäure ihre hemmende Wirkung auf die übrigen Oxydationsprozesse maskiert. Bei Extraktversuchen tritt also die bei Schnittversuchen nur angedeutete Verbrennung der Mandelsäure deutlicher in Erscheinung. Die Ursache hierfür könnte sein, dass das Gewebe bei Schnittversuchen mehr Nährstoffe zur Verfügung hat, und dass sich deshalb die Anwesenheit der Mandelsäure in den Schnittversuchen weniger stark geltend macht als in Extraktversuchen.

Aus den Diagrammen 12 und 13 geht hervor, dass in Versuchen mit Nierenextrakt die *spontane Ammoniakbildung durch Benzoesäure oder Mandelsäure* in einer der Konzentrationen zwischen $0{,}78 \times 10^{-3}$ Mol/l und 0,1 Mol/l *nicht gehemmt* zu werden scheint (siehe auch Diagramm 15). Dies ist ein bemerkenswerter Gegensatz zu den Verhältnissen im Nierenschnitt. Zur Erklärung kann man sich denken, die Ammoniakbildung im Nierenschnitt und die im Nierenextrakt seien zum Teil verschiedenen Ursprungs. Wenn man dabei bedenkt, dass — was schon früher (Seite 18) gesagt wurde — im Extrakt keine l-Aminosäuren abgebaut werden, erscheint dieser Gedanke haltbar, und, soviel wir wissen, werden ja unter normalen Verhältnissen in Niere und Leber auch keine d-Aminosäuren abgebaut. Es ist also wahrscheinlich, dass die Stoffe, die in den Extraktversuchen die spontane Ammoniakbildung veranlassen, im Gegen-

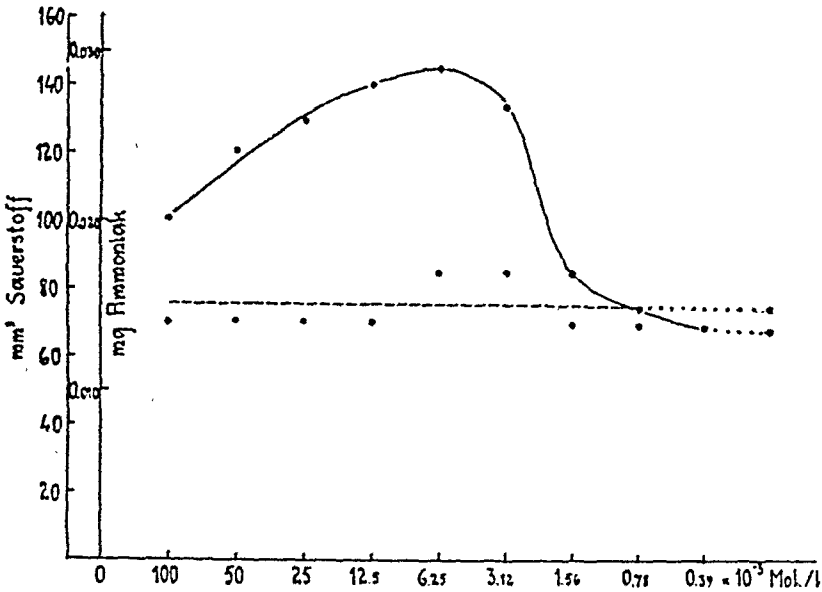


Diagramm 13. Mass des *spontanen Sauerstoffverbrauchs* und der *spontanen Ammoniakbildung* bei Versuchen mit *Nierenextrakt* ohne und bei Anwesenheit von Mandelsäure in verschiedenen Konzentrationen. Die Werte ganz rechts geben die Stärke des Stoffwechsels ohne Mandelsäure an.

Sauerstoffverbrauch. . . . . ●———●
Ammoniakbildung . . . . . ●- - - - -●

satz zu dem, was für Schnittversuche gilt, keine Aminosäuren sind. Der Unterschied, der sich unter den ungleichen Versuchsbedingungen zwischen der Wirkung der Benzoesäure und der der Mandelsäure zeigt, würde dann darauf beruhen, dass verschiedene Enzymsysteme für Vergiftung durch diese Stoffe verschieden empfindlich wären. Dies würde dann dafür sprechen, dass die *Desaminierung der* von Natur aus im Nierengewebe vorkommenden *Aminosäuren* durch Benzoesäure und Mandelsäure *gehemmt* wird. Die Frage soll später näher behandelt werden.

Leberextrakt.

Die Versuche wurden nach der auf Seite 24 ff. angegebenen Methode ausgeführt.

Da die geschilderten Versuche mit sowohl Leberschnitten als auch Nierenextrakt zeigen, dass in diesen Fällen weder Benzoesäure noch Mandelsäure die Ammoniakbildung hemmen, besteht Grund zu der Annahme, dass diese auch bei Versuchen mit Leberextrakt nicht gehemmt werde. Deshalb wurden die Versuche mit Leberextrakt auf die Untersuchung der Wirkung von *Benzoesäure* in einer Kon-

zentration von 0,025 Mol/l eingeschränkt. Die Ergebnisse, die aus Tabelle 5 ersichtlich sind, zeigen, dass der *Sauerstoffverbrauch* etwas *gehemmt* wird, während *keine* sichere *Hemmung der Ammoniakbildung* auftritt (siehe auch Diagramm 17).

TABELLE 5.

Die von einer 0,025-mol Lösung von Benzoesäure auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Leberextrakt ausgeübte Hemmung.

Stoff	Sauerstoffverbrauch		Ammoniakbildung	
	Hemmung in %	Mittelwert	Hemmung in %	Mittelwert
Benzoesäure	16 10	13	5 5	5

Zusammenfassung der Ergebnisse.

Aus den Untersuchungen dürften folgende Hauptschlüsse gezogen werden können: *1.* Dass Benzoesäure und Mandelsäure den Sauerstoffverbrauch in Versuchen mit Nieren- und Leber-Schnitt hemmen. Die durch die Benzoesäure ausgeübte Hemmung scheint dabei etwas stärker zu sein, aber der Unterschied dürfte nicht auf tatsächlich verschieden starker Hemmungswirkung beruhen, sondern darauf, dass Mandelsäure gleichzeitig verbrannt wird. *2.* Dass Benzoesäure und Mandelsäure die Ammoniakbildung im Nierenschnitt gleich stark hemmen, dass aber im Leberschnitt die Ammoniakbildung offenbar nicht gehemmt wird. *3.* Dass der Sauerstoffverbrauch im Nierenextrakt durch Benzoesäure gehemmt, durch Mandelsäure aber stimuliert wird, was wahrscheinlich darauf beruht, dass letztere verbrannt wird. *4.* Dass die Ammoniakbildung im Nierenextrakt weder von Benzoesäure noch von Mandelsäure gehemmt wird. *5.* Dass der Sauerstoffverbrauch im Leberextrakt von Benzoesäure anscheinend etwas gehemmt wird, während die Ammoniakbildung nicht beeinflusst wird.

Hieraus ergibt sich also, dass kein Anhaltspunkt für die frühere Vermutung (Seite 7) zu finden ist, die Mandelsäure übe im Zusammenhang mit ihrem Bau als α-Oxysäure eine spezielle hemmende Wirkung auf die Aminosäuredesaminierung aus.

Der Einfluss von mit Benzoesäure und Mandelsäure verwandten Stoffen auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Nieren- und Leber-Gewebe.

Oben ergab sich, dass der graduelle Hemmungsunterschied bei der Wirkung von Benzoesäure und Mandelsäure nicht auf eine prinzipielle Verschiedenheit der Wirkungsweise deutet; d. h. die Ungleichheit der Konstitution, wie sie bei diesen beiden Stoffen vorliegt, scheint keine nachweisbare Rolle zu spielen. Bei Stoffen mit grösserer Unähnlichkeit im Aussehen der Moleküle besteht jedoch Grund zu der Erwartung, dass eine irgendwie andersgeartete Hemmungswirkung auftrete. Um zu erfahren, *welche Gruppe* im Molekül, hier der Benzoesäure und dort der Mandelsäure, *die aktive sei*, wurden daher, in einer im übrigen mit der der früher beschriebenen Versuche analogen Weise, auch Versuche mit Stoffen angestellt, die in ihrer Konstitution von diesen beiden Säuren auf verschiedene Art abweichen.

Die Versuche wurden an *Nieren- und Leber-Schnitten* nach der auf Seite 24 ff. angegebenen Methode ausgeführt. Die *Konzentration der untersuchten Stoffe* im Reaktionsgemisch war dabei durchgehends *0,025 Mol/l*.

Die Ergebnisse der Nierenschnitt-Versuche sind in Tabelle 6 und die der Leberschnitt-Versuche in Tabelle 7 zusammengestellt, wobei die untersuchten Stoffe nach ihrer konstitutionellen Gleichheit in Gruppen geordnet wurden. Um ein besseres Verständnis der Vergleiche zu ermöglichen, und weil die Variationen der Hemmungswirkung verschiedener Stoffe in vielen Fällen gering sind, wurde das Verhältnis zwischen dem Stoffwechsel bei Vorhandensein und dem bei Fehlen der untersuchten Stoffe nicht nur in Prozenten angegeben, sondern es wurden für jeden Versuch auch die absoluten Zahlen für die Stärke des unter verschiedenen Bedingungen herrschenden Stoffwechsels in die Tabellen eingetragen.

1. Gruppe. *Natriumchlorid.*

Diese Versuche zeigen die Stärke der unspezifischen Salzwirkung. Sie wurden schon auf Seite 41 besprochen.

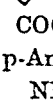
2. Gruppe. *Benzoesäure, Phenylessigsäure, β-Phenylpropionsäure.*

Mit Hilfe dieser Stoffe wurde der Einfluss der Länge der Fettsäurekette bei den phenylsubstituierten Fettsäuren studiert.

TABELLE 6.

Einwirkung von Benzoesäure und verwandten Stoffen in 0,025-mol Lösung auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Nierengewebe.

Stoffe	Sauerstoffverbrauch in mm³ während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %		Ammoniakbildung in mg während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %	
	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert
1	2	3	4	5	6	7	8	9
1. *Gruppe.*								
Natriumchlorid[1])	282	252	—11		0,026	0,024	—8	
NaCl	300	283	—6	—10	0,031	0,029	—6	—9
	282	247	—12		0,025	0,022	—12	
	315	276	—12		0,029	0,026	—10	
2. *Gruppe.*								
Benzoesäure[1])	251	77	—69		0,029	0,014	—52	
⬡	267	99	—63	—66	0,030	0,014	—53	—52
	214	96	—55		0,024	0,013	—46	
COOH	230	58	—75		0,027	0,011	—59	
Phenylessigsäure[3])	281	92	—67	—68	0,029	0,009	—55	—58
⬡	284	86	—70		0,018	0,007	—61	
CH_2COOH								
β-Phenylpropionsäure[4]) ...	268	43	—84	—82	0,030	0,015	—50	—56
⬡	290	62	—79		0,026	0,010	—62	
CH_2CH_2COOH								
3. *Gruppe.*								
Ameisensäure[1])	260	202	—22		0,021	0,017	—19	
HCOOH	286	211	—26	—26	0,021	0,018	—14	—19
	260	180	—31		0,017	0,013	—24	
Essigsäure[5])	346	357	+3	—1	0,029	0,027	—7	—12
CH_3COOH	303	288	—5		0,022	0,018	—18	
Propionsäure[2])	258	164	—36		0,018	0,014	—22	
CH_3CH_2COOH	231	147	—36	—37	0,019	0,015	—21	—23
	247	147	—40		0,019	0,014	—26	
Buttersäure[5])	291	285	—2	—3	0,023	0,017	—26	—22
$CH_3CH_2CH_2COOH$.	285	273	—4		0,027	0,022	—18	
4. *Gruppe.*								
Mandelsäure[6])	307	188	—39					
⬡	267	195	—27		0,025	0,012	—52	
	251	138	—45	—39	0,029	0,016	—45	—50
CHOHCOOH	281	170	—40		0,020	0,011	—45	
	284	160	—44		0,018	0,008	—56	

Stoffe	Sauerstoffverbrauch in mm³ während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %		Ammoniakbildung in mg während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %	
	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert
1	2	3	4	5	6	7	8	9
Phenylglyoxylsäure[7])	295	79	—73	—72	0,018	0,004	—78	—61
	278	83	—70		0,018	0,008	—56	
COCOOH	286	80	—72		0,025	0,013	—48	
5. *Gruppe.*								
Hippursäure[2])	215	143	—33	—43	0,017	0,011	—35	—27
	299	167	—44		0,020	0,016	—20	
$CONHCH_2COOH$	275	132	—52		0,026	0,019	—27	
6. *Gruppe.*								
o-Oxybenzoesäure (Salicylsäure)[3])	292	42	—86	—85	0,020	0,008	—60	—61
	270	44	—84		0,019	0,008	—58	
OH	279	42	—85		0,022	0,008	—64	
COOH								
m-Oxybenzoesäure[4])	276	100	—64	—61	0,020	0,010	—50	—47
	257	98	—62		0,019	0,011	—42	
OH	249	109	—56		0,018	0,009	—50	
COOH								
p-Oxybenzoesäure[4])	243	109	—55	—59	0,019	0,010	—47	—48
OH	238	91	—62		0,021	0,011	—48	
	248	97	—61		0,024	0,012	—50	
COOH								
7. *Gruppe.*								
o-Aminobenzoesäure[5])	239	111	—54	—57	0,018	0,012	—33	—31
	200	83	—58		0,022	0,016	—27	
NH_2	281	118	—58		0,021	0,014	—33	
COOH								
m-Aminobenzoesäure[2]) ...	267	122	—54	—52	0,019	0,014	—26	—22
	277	137	—50		0,019	0,014	—26	
NH_2	246	121	—51		0,020	0,017	—15	
COOH								
p-Aminobenzoesäure[2])	230	99	—57	—54	0,021	0,016	—24	—30
NH_2	251	112	—55		0,015	0,010	—33	
	235	116	—51		0,018	0,012	—33	
COOH								

Stoffe	Sauerstoffverbrauch in mm³ während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %		Ammoniakbildung in mg während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %	
	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert
1	2	3	4	5	6	7	8	9
8. *Gruppe.*								
Benzamid[7])	266	97	—64		0,020	0,014	—30	
⬡	280	117	—58	—59	0,027	0,022	—18	—26
$CONH_2$	230	101	—56		0,017	0,012	—29	
Acetamid[2])	231	218	—6		0,017	0,019	+12	
CH_3CONH_2	234	209	—11	—8	0,019	0,020	+5	+8
Propionamid[7])	271	252	—7		0,022	0,024	+9	
$CH_3CH_2CONH_2$	241	207	—14	—8	0,022	0,025	+14	+18
	211	206	—2		0,019	0,025	+32	
9. *Gruppe.*								
Phenol[3])	231	30	—87		0,018	0,008	—56	
⬡	243	26	—89	—88	0,022	0,012	—45	—48
OH	244	32	—87		0,019	0,011	—42	
Anilinhydrochlorid[5])	239	136	—43	—37	0,016	0,010	—38	—38
⬡	215	149	—31		0,016	0,010	—38	
$NH_2 \cdot HCl$								
10. *Gruppe.*								
Benzolsulfosäure[8])	239	197	—18		0,024	0,018	—25	
⬡	211	178	—16	—18	0,029	0,026	—10	—17
SO_2OH	261	209	—20		0,018	0,015	—17	
Sulfanilsäure[1])	237	218	—8		0,014	0,014	0	
NH_2	235	218	—7	—8	0,018	0,018	0	0
⬡								
SO_2OH								
Sulfanilamid[8])	237	195	—18		0,020	0,019	—5	
NH_2	215	173	—20	—19	0,020	0,020	0	—2
⬡								
SO_2NH_2								

[1]) p. analysi, Merck. [2]) f. wiss. Zw., Merck. [3]) hier gereinigt. [4]) rein, Heyl & Co. [5]) puriss., Merck. [6]) f. wiss. Zw., Schering-Kahlbaum. [7]) hier synthet. u. gereinigt. [8]) synthet. von A/B Pharmacia.

TABELLE 7.

Einwirkung von Benzoesäure und verwandten Stoffen in 0,025-mol Lösung auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Lebergewebe.

Stoffe	Sauerstoffverbrauch in mm³ während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %		Ammoniakbildung in mg während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %	
	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert
1	2	3	4	5	6	7	8	9
1. *Gruppe.*								
Natriumchlorid	263	243	—8	—4	0,019	0,018	—5	—5
	204	204	0		0,019	0,018	—5	
2. *Gruppe.*								
Benzoesäure	247	132	—46	—48	0,029	0,027	—7	—6
	168	86	—49		0,021	0,020	—5	
Phenylessigsäure	208	143	—31	—28	0,028	0,025	—11	—10
	259	191	—26		0,029	0,026	—10	
β-Phenylpropionsäure	244	137	—44	—43	0,028	0,023	—18	—22
	253	147	—42		0,027	0,020	—26	
3. *Gruppe.*								
Ameisensäure	220	218	—1	0	0,025	0,026	+4	+2
	217	216	0		0,025	0,025	0	
Essigsäure	245	274	+12	+5	0,018	0,019	+6	+3
	227	223	—2		0,020	0,020	0	
Propionsäure	137	146	+6	+6	0,015	0,014	—7	—4
	94	100	+6		0,011	0,011	0	
Buttersäure	215	242	+12	+14	0,019	0,019	0	+2
	232	270	+16		0,023	0,024	+4	
4. *Gruppe.*								
Mandelsäure	213	131	—38	—28	0,030	0,029	—3	—4
	240	190	—21		0,024	0,025	+4	
	206	153	—26		0,025	0,022	—12	
Phenylglyoxylsäure	196	135	—31	—28	0,025	0,022	—12	—12
	280	208	—26		0,023	0,020	—13	
5. *Gruppe.*								
Hippursäure	220	205	—7	—8	0,021	0,021	0	+2
	290	241	—17		0,020	0,021	+5	
	292	296	+1		0,019	0,019	0	

Stoffe	Sauerstoffverbrauch in mm³ während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %		Ammoniakbildung in mg während der Versuchsdauer		Hemmung (—) bzw. Stimulierung (+) in %	
	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert	spontan	mit resp. Stoff	im resp. Versuch	Mittelwert
1	2	3	4	5	6	7	8	9
6. *Gruppe.*								
o-Oxybenzoesäure (Salicylsäure)	216	83	—62	—64	0,017	0,014	—18	—14
	232	76	—67		0,021	0,019	—10	
m-Oxybenzoesäure	235	176	—25	—29	0,029	0,030	+3	+1
	254	158	—38		0,029	0,030	+3	
	249	190	—24		0,028	0,027	—4	
p-Oxybenzoesäure	246	151	—39	—36	0,026	0,025	—4	—4
	213	143	—33		0,027	0,026	—4	
7. *Gruppe.*								
o-Aminobenzoesäure	202	155	—23	—22	0,022	0,022	0	0
	208	165	—21		0,026	0,026	0	
m-Aminobenzoesäure	215	146	—32	—26	0,018	0,021	+17	+21
	194	155	—20		0,024	0,030	+25	
p-Aminobenzoesäure	159	124	—22	—22	0,028	0,029	+4	+5
	177	136	—23		0,035	0,037	+6	
8. *Gruppe.*								
Benzamid	218	141	—35	—37	0,020	0,087	+335	+303
	189	134	—29		0,023	0,102	+343	
	173	93	—46		0,019	0,063	+232	
Acetamid	241	226	—6	—3	0,019	0,025	+32	+52
	221	222	0		0,022	0,038	+73	
Propionamid	213	217	+2	+2	0,012	0,025	+108	+108
	160	164	+2		0,012	0,025	+108	
9. *Gruppe.*								
Phenol	185	63	—66	—68	0,012	0,013	+8	+4
	178	53	—70		0,016	0,016	0	
Anilinhydrochlorid	195	180	—8	—14	0,015	0,019	+27	+28
	213	170	—20		0,014	0,018	+28	
10. *Gruppe.*								
Benzolsulfosäure	209	205	—2	—1	0,024	0,023	—4	—2
	221	220	0		0,018	0,018	0	
Sulfanilsäure	214	174	—19	—13	0,019	0,019	0	0
	228	211	—7		0,026	0,026	0	
	168	145	—14		0,021	0,021	0	
Sulfanilamid	249	204	—18	—20	0,014	0,013	—7	—4
	242	186	—23		0,024	0,024	0	

Phenylpropionsäure scheint auf den Sauerstoffverbrauch im Nierengewebe eine etwas stärkere Hemmung auszuüben als Benzoesäure und Phenylessigsäure, während die auf die Ammoniakbildung ausgeübte Hemmung die gleiche zu sein scheint. Im Lebergewebe übt Phenylessigsäure auf den Sauerstoffverbrauch eine etwas geringere Hemmung aus als Benzoesäure und Phenylpropionsäure, die unter sich eine gleich starke Hemmung hervorzurufen scheinen. Möglicherweise liegt bei Verlängerung der Fettsäurekette eine Tendenz zu verstärkter Hemmung der Ammoniakbildung vor. Bei allen drei Stoffen ist die Hemmung sowohl des Sauerstoffverbrauchs wie auch die der Ammoniakbildung im Nierengewebe stärker als im Lebergewebe. Es scheint, als ob die Konstitutionsverschiedenheit bei den untersuchten Stoffen ihre Hemmungswirkung nach keiner Richtung hin stark beeinflusse.

3. Gruppe. *Ameisensäure, Essigsäure, Propionsäure, Buttersäure.*

Die Untersuchung dieser Stoffe ermöglicht einen Vergleich zwischen ihrer Wirkung und der der entsprechenden phenylsubstituierten Säuren der 2. Gruppe; hierdurch könnte die etwaige Rolle der Phenylgruppe bei den zu studierenden Vorgängen vielleicht erkannt werden.

Die Versuche zeigen, dass der Sauerstoffverbrauch im Nierengewebe durch Ameisensäure und Propionsäure, bei denen die Anzahl der Kohlenstoffatome ungerade ist, mässig gehemmt wird, während er von der Essigsäure und der Buttersäure, bei denen die Zahl der Kohlenstoffatome eine gerade ist, nicht beeinflusst zu werden scheint. Die Ammoniakbildung im Nierengewebe scheint dagegen von allen in Rede stehenden Stoffen ziemlich gleichermassen gehemmt zu werden. Dies dürfte darauf deuten, dass die Fettsäuren, die eine gerade Zahl von Kohlenstoffatomen aufweisen, in einem solchen Ausmass verbrannt werden, dass die von ihnen ausgeübte Hemmung maskiert wird: ein Umstand ähnlicher Art wie der bereits (Seite 45) im Zusammenhang mit der Hemmungswirkung der Mandelsäure besprochene. Im Lebergewebe lässt sich eine Wirkung der Stoffe nicht sicher nachweisen. Möglicherweise liegt bei Anwesenheit von Buttersäure eine leichte Stimulation der Verbrennung vor.

Ein Vergleich der Wirkung, die einerseits von den Stoffen in Gruppe 2 und anderseits denen in Gruppe 3 ausgeübt wird, zeigt also, dass *die Fettsäuren eine gewisse hemmende Wirkung* auf den

Stoffwechsel im Nierengewebe auszuüben scheinen, und dass sich *diese Wirkung durch Phenylsubstitution verstärkt.* Im Lebergewebe tritt eine Hemmungswirkung erst dann auf, wenn es sich um Fettsäuren handelt, die eine Phenylgruppe in das Molekül aufgenommen haben.

4. Gruppe. *Mandelsäure, Phenylglyoxylsäure, (Phenylalanin).*

Die Untersuchung dieser Gruppe zeigt, welchen Einfluss eine Substitution in der Fettsäurekette auf die Hemmungswirkung des betreffenden Stoffes hat.

Im vorhergehenden wurde schon die Hemmungswirkung besprochen, die die Mandelsäure — Phenyloxyessigsäure — hervorruft. Damals (Seite 50) wurde darauf hingewiesen, dass sie im Prinzip die gleiche Wirkung wie Benzoesäure haben dürfte, die ihrerseits, im grossen und ganzen gesehen, hinsichtlich ihrer Wirkung mit der Phenylessigsäure übereinstimmt. Ein Vergleich zwischen der Wirkung von einerseits Phenylglyoxylsäure — Phenylketoessigsäure — und anderseits von Phenylessigsäure zeigt ebenfalls, dass die Wirkung der beiden Stoffe grosse Ähnlichkeit hat. Es scheint also, als *spielten* die betreffenden *Substitutionen in der Fettsäurekette keine Rolle* bei der Frage der Hemmungswirkung.

Die Untersuchungen über das Phenylalanin — Phenylaminopropionsäure —, haben keinen Bescheid geliefert, da der Stoff selbst desaminiert wird und dadurch die Feststellung einer eventuellen Hemmung der Ammoniakbildung im Gewebe unmöglich macht. Schon bei einer Konzentration von 0,00125 Mol/l stimulierte d (+) Phenylalanin die Ammoniakbildung bei je zwei Versuchen mit Nierenschnitt resp. Leberschnitt durchschnittlich um 62 % und 70 %.

5. Gruppe. *Hippursäure.*

In diesem Zusammenhang wurde auch die Hippursäure untersucht; nicht wegen ihrer Konstitution, sondern auf Grund ihrer hinsichtlich des Stoffwechsels herrschenden Beziehungen zur Benzoesäure.

Im Nierengewebe scheint die Hippursäure auf sowohl Sauerstoffverbrauch als auch Ammoniakbildung eine geringere Hemmung auszuüben als Benzoesäure, und im Lebergewebe kann eine Hemmung nicht sicher nachgewiesen werden. Es ist interessant zu sehen, wie die starke Hemmungswirkung der Benzoesäure durch ihre Kop-

pelung mit Glykokoll zu Hippursäure anscheinend reduziert wird, was dafür spricht, dass hier eine entgiftende Wirkung vorliegt.

6. Gruppe. *o-Oxybenzoesäure (Salicylsäure), m-Oxybenzoesäure, p-Oxybenzoesäure.*

Die Untersuchungen, die mit den Stoffen in dieser und der nächsten Gruppe angestellt wurden, beabsichtigen, die Wirkung der Substitution eines Wasserstoffatoms am Ring der Benzoesäure zu zeigen.

Die drei verschiedenen Oxybenzoesäuren zeigen in ihrer Wirkung keine völlige Übereinstimmung. Die o-Oxybenzoesäure scheint durchgehends eine etwas kräftigere Hemmung auszuüben als die beiden andern, die untereinander eine fast ganz übereinstimmende Wirkung aufweisen, die ihrerseits auch mit der der Benzoesäure ziemlich übereinstimmt. Im Lebergewebe scheint jedoch die Benzoesäure eine etwas kräftigere Hemmung auf den Sauerstoffverbrauch auszuüben als m- und p-Oxybenzoesäure. Die Versuche scheinen also zu zeigen, dass *durch die Substitution in Orthostellung die Hemmungswirkung* im Vergleich zu der der Benzoesäure *verstärkt* wird.

7. Gruppe. *o-Aminobenzoesäure, m-Aminobenzoesäure, p-Aminobenzoesäure.*

Die drei Aminobenzoesäuren, die bei Substitution eines Wasserstoffatoms am Ring der Benzoesäure durch eine Aminogruppe entstehen, üben auf den Stoffwechsel im Nierengewebe eine ziemlich gleichgrosse Hemmung aus. Auch im Lebergewebe üben alle eine ungefähr gleichgrosse Hemmung auf den Sauerstoffverbrauch aus, während die m-Aminobenzoesäure im Gegensatz zu den anderen die Ammoniakbildung etwas zu stimulieren scheint. Verglichen mit Benzoesäure üben sie im Nierengewebe alle eine geringere Hemmung auf die Ammoniakbildung aus, und auch beim Sauerstoffverbrauch liegt möglicherweise eine Tendenz zu schwächerer Hemmung vor. Im Lebergewebe üben sie eine geringere Hemmung auf den Sauerstoffverbrauch aus als Benzoesäure. Die Versuche scheinen also zu zeigen, dass durch die in Rede stehende *Substitution die Hemmungswirkung* im Vergleich zu derjenigen der Benzoesäure *etwas gemindert* wird.

Die Ergebnisse der Untersuchungen über die Stoffe der 4., 6. und 7. Gruppe zusammenfassend, kann also gesagt werden, dass keine der hier besprochenen *Substitutionen am Ring oder an der Kette eine wesentliche Veränderung der Hemmung* des Sauerstoffverbrauchs und der Ammoniakbildung im Nieren- und Leber-Gewebe mit sich

zu bringen scheint. Bemerkenswert ist jedoch die Wirkung der Salicylsäure; diese scheint nämlich durchgehends eine etwas kräftigere Hemmung auszuüben als die Benzoesäure.

8. Gruppe. *Benzamid*, (*Formamid*), *Acetamid*, *Propionamid*. Die Untersuchung der Stoffe in dieser Gruppe beabsichtigt, die Wirkung zu studieren, die eine Substitution in der Carboxylgruppe der Benzoesäure und der niedrigeren Fettsäuren auf die Hemmungswirkung der betreffenden Stoffe ausübt. So kann die Frage untersucht werden, welche Rolle die freie Carboxylgruppe bei den in Betracht gezogenen Vorgängen spielt.

Benzamid scheint auf den Sauerstoffverbrauch im Nierengewebe eine gleichstarke Hemmung auszuüben wie Benzoesäure. Formamid konnte nicht untersucht werden, da es ohne Anwesenheit von Gewebe spontan Ammoniak abgibt. Acetamid übt, wie Essigsäure, keine sichere Hemmung auf den Sauerstoffverbrauch im Nierengewebe aus, und das Gleiche gilt, im Gegensatz zur Propionsäure, für Propionamid. Auf die Ammoniakbildung im Nierengewebe übt das Benzamid eine geringere Hemmung aus als die Benzoesäure, und Acetamid und Propionamid zeigen eher eine Tendenz zur Stimulation der Ammoniakbildung, im Gegensatz zu Essigsäure und Propionsäure, die sie leicht zu hemmen scheinen. Die besagte Stimulation kann indessen vielleicht durch Verunreinigungen in den Präparaten verursacht sein. Im Lebergewebe scheint Benzamid eine ungefähr ebensogrosse Hemmung des Sauerstoffverbrauches auszuüben wie die Benzoesäure, und Acetamid und Propionamid scheinen wie die Essigsäure und die Propionsäure indifferent zu sein. Auf die Ammoniakbildung im Lebergewebe wirken alle drei Amide stimulierend, was besonders beim Benzamid kräftig in Erscheinung tritt. Dies dürfte bedeuten, dass die Stoffe selbst desamidiert werden. Da auf diese Weise die Hemmungswirkung dieser Stoffe auf die Ammoniakbildung überdeckt wird, ist es schwer, auf die Stärke der Hemmung zu schliessen. Jedoch scheint es, als liege, im Vergleich zur freien Carboxylgruppe, *bei* der einer *Aminosubstitution* ausgesetzten Carboxylgruppe eine *Tendenz zur Minderung der Hemmungswirkung* vor.

Im Folgenden (Seite 84) soll die Einwirkung der Benzoesäure auf die verantwortlichen desamidierenden Enzyme untersucht werden wie auch die Wirkung auf einige andere Enzyme, die eine Ammoniakbildung im Nieren- resp. Leber-Gewebe vermitteln.

9. Gruppe. (*Benzol*), *Phenol*, *Anilin*.

Mit Hilfe dieser Stoffe soll die Wirkung des Benzolringes bei Fehlen der Carboxylgruppe untersucht werden.

Das Benzol selbst konnte nicht untersucht werden, da es in zu geringem Grade wasserlöslich ist. Phenol hemmt den Sauerstoffverbrauch im Nierengewebe erheblich stärker als Anilin, während der Unterschied in der Wirkung der beiden Stoffe auf die Ammoniakbildung nicht so signifikativ ist. Im Lebergewebe hemmt Phenol auch den Sauerstoffverbrauch stark, während die Wirkung des Anilins unbedeutend ist. Auf die Ammoniakbildung im Lebergewebe dagegen übt Phenol keine Wirkung aus, während Anilin die Ammoniakbildung ein wenig zu stimulieren scheint. Am nächsten liegt da der Gedanke, das Ammoniak stamme aus der Aminogruppe. Verglichen mit der Benzoesäure hemmt das Phenol den Sauerstoffverbrauch sowohl im Nieren- als auch im Leber-Gewebe stärker, während die Hemmung der Ammoniakbildung durch die beiden Stoffe die gleiche zu sein scheint. Das deutet darauf, dass das Phenol auch solche Oxydationsprozesse stark hemmt, bei deren Verlauf kein Ammoniak freiwird. Anilin scheint durchweg eine schwächere Wirkung als Benzoesäure zu haben.

Es ergibt sich also, dass *der Benzolring, auch wenn keine Carboxylgruppe*, sondern eine Hydroxylgruppe oder Aminogruppe *angegliedert ist, eine Hemmung* auf den Stoffwechsel im Nieren- und Lebergewebe *ausübt*. Diese Hemmung scheint im ersten der beiden Fälle kräftiger, im zweiten schwächer zu sein als die durch Benzoesäure hervorgerufene.

10. Gruppe. *Benzolsulfosäure*, *Sulfanilsäure*, *Sulfanilamid*.

Die Stoffe dieser Gruppe enthalten eine Sulfosäuregruppe an Stelle einer Carboxylgruppe.

Im Gegensatz zu dem, was bei den entsprechenden Carbonsäuren, der Benzoesäure und der p-Aminobenzoesäure, der Fall war, zeigen die Versuche, dass die Stoffe durchgehends eine sehr geringe oder keine Wirkung auf den Grad des Sauerstoffverbrauchs und der Ammoniakbildung im Nieren- und Leber-Gewebe haben. Es scheint also, als hebe die Sulfosäuregruppe die Giftwirkung, die sich beim Benzolring in Verbindung mit anderen Gruppen gezeigt hatte, beinahe oder ganz auf.

Zusammenfassung der Ergebnisse.

Als hauptsächliche Schlussfolgerungen aus den hier wiedergegebenen Untersuchungen dürfte sich Folgendes zusammenfassen lassen. *1.* Dass die phenylsubstituierten Fettsäuren eine offenbar erheblich stärkere Hemmungswirkung auf den Sauerstoffverbrauch und die Ammoniakbildung im Nierengewebe und auf den Sauerstoffverbrauch im Lebergewebe ausüben als die entsprechenden Fettsäuren. *2.* Dass anscheinend die Länge der Fettsäurekette in den phenylsubstituierten Säuren keine grössere Rolle spielt. *3.* Dass eine Substitution an der Kette bei Phenylessigsäure (zu Mandelsäure oder Phenylglyoxylsäure) oder im Ring der Benzoesäure (zu einer der Oxy- oder Aminobenzoesäuren) die Hemmungswirkung nicht wesentlich verändert; die Salicylsäure scheint jedoch eine durchgehends kräftigere Hemmungswirkung auszuüben als die Benzoesäure selbst. *4.* Dass der Benzolring im Verein mit einer Hydroxyl-, Amino- oder Säureamid-Gruppe, jedoch ohne Carboxylgruppe, eine Hemmung ausübt, die der Stärke nach anscheinend nicht erheblich von der durch die Benzoesäure ausgelösten abweicht. *5.* Dass die Sulfosäuregruppe fast völlig die Hemmungswirkung aufzuheben scheint, die der Benzolring in Verbindung mit den obengenannten Gruppen ausübt.

Die Fragestellung in den eben besprochenen Untersuchungen war: Welches ist, hinsichtlich deren nachgewiesener Hemmungswirkung, *die aktive Gruppe* im Molekül der Benzoesäure und der Mandelsäure? Aus den Untersuchungen scheint hervorzugehen, dass es *die Kombination der Phenyl- und Carboxylgruppe* ist, die diese Wirkung bedingt. Denn beide Gruppen, je für sich, scheinen zwar in der Regel eine Hemmung hervorzurufen, aber diese ist gewöhnlich schwächer als die, welche die beiden Gruppen hervorrufen, wenn sie in phenylsubstituierten Fettsäuren gemeinsam auftreten.

Weiterhin war es interessant, die Ergebnisse dieser Untersuchungen mit ähnlichen zu vergleichen, die von Anderen angestellt wurden.

MÅHLÉN (1928) findet in seiner oben (Seite 11) angezogenen Untersuchung über den Einfluss der Benzoesäure, der p-Oxybenzoesäure, der m-Oxybenzoesäure und der Salicylsäure auf die Succinodehydrase, dass diese Stoffe eine nach der Aufzählungsordnung an Stärke zunehmende Hemmung ausüben.

Auch in vorliegender Untersuchung hat die Salicylsäure eine stärkere Hemmungswirkung gezeigt als die übrigen ebengenannten

Säuren; zwischen diesen letzten konnte jedoch ein sicherer Unterschied nicht nachgewiesen werden.

KLEIN o. KAMIN (1941) haben bei ihren Versuchen über die von der Benzoesäure ausgeübte Hemmung auf die d-Aminosäureoxydase (Seite 11) auch die Wirkung einiger anderer Stoffe untersucht und mit der der Benzoesäure verglichen. Sie fanden dabei, dass die Carboxylgruppe notwendig war, um eine Hemmung zu erzeugen, ferner dass der Benzolring effektiver war als andere Ringe, sowie dass eine Substitution am Ring oder zwischen diesem und der Carboxylgruppe die Hemmungswirkung verminderte. Die Hemmung der d-Aminosäureoxydase scheint also derjenigen bei der spontanen Gewebeoxydation und bei der spontanen Ammoniakbildung im Nieren- und Leber-Gewebe nicht ganz gleichwertig zu sein.

Auch Untersuchungen von v. EULER (1942) zeigen (Seite 12), dass hinsichtlich der Frage nach der Hemmungswirkung der Benzoesäure und ihr verwandter Stoffe auf Milchsäuredehydrase die Verhältnisse etwas anders liegen als im Falle der spontanen Gewebeoxydation und der spontanen Ammoniakbildung. So findet v. EULER, dass Salicylsäure eine erheblich kräftigere Wirkung als Benzoesäure ausübt, während diese ungefähr die gleiche Wirkung hat wie Benzamid. Dagegen hat laut v. EULER Sulfanilsäure eine stärkere Wirkung als Benzoesäure.

Die Benzoesäure und ihr verwandte Stoffe hemmen also eine Anzahl verschiedener Enzymsysteme, aber die Stoffe scheinen dabei auf die Systeme verschieden stark zu wirken.

Der Einfluss der Benzoesäure auf verschiedene ammoniakbildende Prozesse im Nieren- und Leber-Gewebe.

Um nun den Einfluss der im vorhergehenden untersuchten Stoffe auf verschiedene ammoniakbildende Prozesse im Nieren- und Leber-Gewebe zu untersuchen, wurde in den folgenden Versuchen *Benzoesäure als* ihr *Repräsentant* verwendet. Da die Wirkung der Benzoesäure bei den früheren Untersuchungen von der verschiedener anderer untersuchter Stoffe nicht wesentlich abweicht, und da die Benzoesäure im Zentrum des Interesses steht, schien es richtig zu sein, gerade sie für die besagte Rolle zu wählen.

Aus den früher geschilderten Versuchen ging hervor, dass durch die verschiedenen hemmenden Stoffe die Ammoniakbildung nur im

Nierengewebe wesentlich gehemmt wird während sie im Lebergewebe nicht sicher beeinflusst wird. Deshalb sind natürlich vor allem die Versuche mit Nierengewebe von grösserem Interesse. Sämtliche Untersuchungen, von denen in diesem Kapitel die Rede sein wird, wurden jedoch auch an Lebergewebe vorgenommen, und auch dies hat Aufschlüsse von positivem Interesse gegeben.

Es ist denkbar, dass die Hemmungswirkung, die von Benzoesäure und den anderen Stoffen auf die spontane Ammoniakbildung im Nierengewebe ausgeübt wird, eine *Giftwirkung* ist, *die alle ammoniakbildenden Prozesse* im Gewebe *in gleich hohem Masse betrifft.* Anderseits kann man sich aber auch vorstellen, dass nur *gewisse Prozesse beeinflusst* werden, während dies bei anderen garnicht oder doch in weit geringerem Mass der Fall ist. Bei einer Benzoesäurekonzentration von 0,025 Mol/l wird zum Beispiel die spontane Ammoniakbildung um ca. 50 % gehemmt (Tabelle 6); dies könnte also entweder darauf beruhen, dass jederlei Ammoniakbildung auf die Hälfte herabgesetzt wird, oder aber darauf, dass einer oder mehrere ammoniakbildende Prozesse um mehr als 50 %, einer oder mehrere andere aber um weniger als 50 % gehemmt werden und zwar in solcher Weise, dass sich eine durchschnittliche Hemmung von ca. 50 % ergibt. Ein Versuch zur Klärung dieses Problems wird dadurch unternommen werden, dass der Einfluss der Benzoesäure auf die von verschiedenen Ammoniakspendern stimulierte Ammoniakbildung untersucht wird.

Die Versuche wurden nach der auf Seite 24 ff. beschriebenen Methode ausgeführt. Die Ergebnisse wurden in den Tabellen 8 und 9 sowie 11 und 12 zusammengestellt. Hier wurde die während der Versuchsdauer, teils in Spontanversuchen, teils bei Anwesenheit eines Substrates und ferner noch die bei Anwesenheit von sowohl Substrat als auch Benzoesäure gebildete absolute Ammoniakmenge angegeben. Die Substratkonzentration war variiert worden, während die der Benzoesäure konstant 0,025 Mol/l entsprach.

Bei Versuchen mit Nieren- und Leber-Extrakt sowie Leberschnitten übt Benzoesäure von 0,025-mol Konzentration keine sichere Hemmung auf die spontane Ammoniakbildung aus (Diagramme 15, 17 und 6). Dagegen *hemmt Benzoesäure bei Nierenschnitt-Versuchen die spontane Ammoniakbildung um ca. 50 %*, was aus früheren Versuchen (Diagramm 2, Tabelle 6) hervorging. Dies muss also berücksichtigt werden bei der Berechnung der Hemmungswirkung, die die Benzoesäure auf die Ammoniakbildung ausübt, die von dem zugesetzten Substrate ausgeht. Leider reicht nur in Ausnahmefällen die Schnittmenge von

den Nieren eines und desselben Tieres zu den 8 Einzelversuchen, die nötig sind, wenn die Hemmungswirkung der Benzoesäure auf die spontane Ammoniakbildung bei jedem Versuch gleichfalls untersucht werden soll. Gewöhnlich reichen die Schnitte nur zu höchstens 6 Versuchen, wenn jeder Einzelversuch an 60 mg Schnittmenge gemacht werden soll. Bei einer Verminderung der Gewebemenge für den einzelnen Versuch, die ein sicheres Ausreichen der verfügbaren Gesamtmenge für 8 Einzelversuche gewährleisten würde, entsteht die Gefahr einer grösseren Ungenauigkeit, was aus den auf Seite 32 angeführten Versuchen hervorgeht. Wenn es auch nicht einwandfrei ist, schien es deshalb doch am richtigsten zu sein, die Versuche wie gewöhnlich an 60 mg Schnittmenge auszuführen und lieber die zwei Einzelversuche auszuschliessen, die der Hemmungswirkung der Benzoesäure auf die spontane Ammoniakbildung gelten. Bei der Berechnung wurde die Hemmung dann als 50 % betragend angenommen; diese Zahl liegt nahe dem mittleren Wert für die 4 Versuche (Tabelle 6), die über die Hemmungswirkung einer 0,025-mol Benzoesäurelösung auf die spontane Ammoniakbildung im Nierenschnitt angestellt wurden. Um die Ergebnisse der Nierenschnittversuche, die dabei in höheren Grade als andere nur als Annäherungswerte aufzufassen sind, zu sichern, wird in jedem Versuch, bei dem es für die Schlussfolgerungen von Bedeutung sein kann, angegeben werden, innerhalb welcher Grenzen das Resultat variiert, wenn die *Hemmung der spontanen Ammoniakbildung zwischen 40 und 60 % variiert.* In den angegebenen Versuchen hat sie zwischen 46 und 59 % variiert. Auf den Tabellen 8 und 11, Spalte 8, sind also die Werte für Versuche mit Nierenschnitten nach einer als 50 % betragend angenommenen Hemmung auf die spontane Ammoniakbildung errechnet.

In den Tabellen 8, 9, 11 und 12 wurden nur die Werte für das Mass der Ammoniakbildung, *nicht* aber die für die *Grösse des Sauerstoffverbrauchs angegeben.* Letztere erbieten bei diesen Versuchen ein geringeres Interesse, da die untersuchten Substrate im allgemeinen in so niedriger Konzentration zugesetzt worden waren, dass sie den Sauerstoffverbrauch nur unbedeutend beeinflussten, weshalb die dabei gewonnenen Ergebnisse wenig Aufklärung geben.

Der Einfluss der Benzoesäure auf die Desaminierung der l-Aminosäuren.

In erster Linie wurden die l-Aminosäuren untersucht, da sie, was schon früher (Seite 14) hervorgehoben wurde, im Nieren- und Leber-Gewebe die wichtigsten Ammoniakspender sein dürften.

Nach Krebs (1932, 1933 a) wird in In-vitro-Versuchen an Nierengewebe die d-Komponente der verschiedenen Aminosäuren bedeutend schneller abgebaut als die l-Komponente. Hiervon ausgenommen sind eigentlich nur die l (+) Glutaminsäure und die l (—) Asparaginsäure. die nicht bloss schneller als die entsprechenden

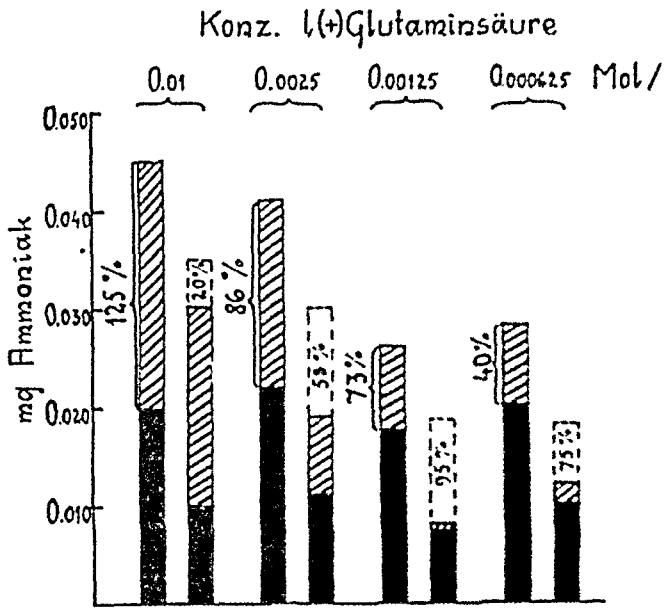


Diagramm 14. *Spontane und von l (+) Glutaminsäure in verschiedenen Konzentrationen stimulierte Ammoniakbildung im Nierenschnitt, sowie Hemmung* der Ammoniakbildung *durch* 0,025-mol *Benzoesäure*lösung. Innerhalb jedes Säulenpaars gibt die Säule links das Mass der Ammoniakbildung ohne Benzoesäure und die Säule rechts dasjenige der Ammoniakbildung mit Benzoesäure an. Der ganzschwarze Teil der Säulen gibt die spontane Ammoniakbildung an, die bei Anwesenheit von Benzoesäure als zur Hälfte reduziert angenommen wird. Der schraffierte Teil der Säulen gibt die durch l (+) Glutaminsäure stimulierte Ammoniakbildung an, an dem die durch Benzoesäure verursachte Hemmung in Prozenten angegeben ist (Weisser Teil der Säulen). Dieses Diagramm wurde mitaufgenommen, um die Berechnung der Werte in den Tabellen 8, 9, 10 und 12 zu erläutern.

d-Komponenten abgebaut werden, sondern bei denen der Abbau auch quantitativ in ziemlich grossem Umfang erfolgt.

Als Repräsentanten für die l-Aminosäuren haben deshalb in erster Linie die beiden genannten gedient. Ausserdem wurden auch l (—) Leucin und l (—) Prolin untersucht. Diese werden allerdings erheblich langsamer als jene abgebaut, sind aber als Repräsentanten für zwei andere konstitutionell verschiedene Typen doch von Interesse.

Aus Tabelle 8 und Diagramm 14 ergibt sich der Grad der Stimulierung der Ammoniakbildung, die durch *l (+) Glutaminsäure verschiedener Konzentration* im *Nierenschnitt* hervorgerufen wird, sowie die gleichzeitige, durch Benzoesäure verursachte Hemmung (siehe auch Diagramm 4). Es zeigt sich, dass sich die Hemmungswirkung

TABELLE 8.

Einwirkung von Benzoesäure in 0,025-mol Lösung auf die Ammoniakbildung aus l- und d-Aminosäuren im Nierengewebe.

Gewebe und Substrat[1])	Substrat-Konz. Mol/l	Ammoniakbildung in mg		Stimulierung in %	Mittelwert	Ammoniakbildung mit Substrat und Benzoesäure in mg	Hemmung der Substratstimulierung durch Benzoesäure in %	Mittelwert
		spontan	mit Substrat					
1	2	3	4	5	6	7	8	9
Nierenschnitt								
l (+) Glutaminsäure	0,01	0,020	0,045	125		0,030	20	
	0,0025	0,022	0,041	86		0,019	58	
	0,00125	0,015	0,026	73		0,008	95	
	0,000625	0,020	0,028	40		0,012	75	
l (—) Asparaginsäure	0,00125	0,018	0,030	67	84	0,000	100	95
	0,00125	0,015	0,030	100		0,009	90	
l (—) Leucin	0,0025	0,018	0,026	44		0,013	50	
	0,00125	0,023	0,030	30		0,014	64	
	0,000625	0,020	0,023	15	18	0,011	67	71
	0,000625	0,018	0,022	22		0,010	75	
l (—) Prolin	0,00125	0,014	0,018	28	33	0,004	175	142
	0,00125	0,013	0,018	38		0,006	110	
d (—) Alanin	0,00125	0,019	0,060	216		0,046	11	
	0,000625	0,022	0,050	127	100	0,016	82	88
	0,000625	0,015	0,026	73		0,008	95	
d (—) Valin	0,00125	0,018	0,031	72		0,010	92	
	0,000625	0,017	0,025	47		0,009	94	
d (+) Phenylalanin...	0,00125	0,017	0,030	76	62	0,008	104	104
	0,00125	0,021	0,031	48		0,010	105	
Nierenextrakt								
d (—) Alanin	0,0025	0,009	0,042	367	284	0,022	61	68
	0,0025	0,008	0,024	200		0,012	75	
	0,00125	0,014	0,054	286		0,018	90	
	0,000625	0,013	0,026	100		0,014	92	
d (—) Valin	0,00125	0,008	0,016	100	106	0,006	125	107
	0,00125	0,016	0,034	112		0,018	89	
	0,000625	0,011	0,015	36		0,010	125	
d (+) Phenylalanin...	0,00125	0,014	0,036	157	191	0,015	95	94
	0,00125	0,016	0,052	225		0,018	94	

[1]) Die in dieser u. ff. Tab. genannten Substrate sind Präparate für wissensch. Zw. v. Heyl & Co., Merck, Hoffmann-La Roche u. Schering-Kahlbaum.

TABELLE 9.

Einwirkung von Benzoesäure in 0,025-mol Lösung auf die Ammoniakbildung aus l- und d-Aminosäuren im Lebergewebe.

Gewebe und Substrat	Substrat-Konz. Mol/l	Ammoniakbildung in mg		Stimulierung in %	Mittelwert	Ammoniakbildung mit Substrat und Benzoesäure in mg	Hemmung (—) resp. Stimulierung (+) der Substratstimulierung durch Benzoesäure in %	Mittelwert
		spontan	mit Substrat					
1	2	3	4	5	6	7	8	9
Leberschnitt								
l (+) Glutaminsäure	0,01	0,008	0,009	12		0,004	—	
	0,0025	0,010	0,011	10		0,009	—	
l (—) Asparaginsäure	0,01	0,013	0,019	46	51	0,017	—33	—22
	0,01	0,018	0,028	56		0,027	—10	
	0,0025	0,010	0,013	30	28	0,013	0	0
	0,0025	0,015	0,019	27		0,019	0	
l (—) Leucin	0,01	0,018	0,027	50	47	0,026	—11	—39
	0,01	0,008	0,013	62		0,011	—40	
	0,01	0,010	0,013	30		0,011	—67	
	0,0025	0,011	0,014	27		0,012	—67	
l (—) Prolin	0,01	0,019	0,027	42		0,026	—12	
	0,0025	0,012	0,016	33		0,015	—25	
d (—) Alanin	0,00125	0,022	0,066	200	212	0,068	+4	+6
	0,00125	0,013	0,042	223		0,044	+7	
	0,000625	0,011	0,022	100		0,024	+18	
d (—) Valin	0,0025	0,018	0,035	94	64	0,028	—41	—26
	0,0025	0,024	0,032	33		0,031	—12	
	0,000625	0,014	0,016	14		0,015	—50	
d (+) Phenylalanin...	0,00125	0,015	0,025	67	70	0,022	—30	—24
	0,00125	0,015	0,026	73		0,024	—18	
Leberextrakt								
d (—) Alanin	0,0025	0,010	0,023	130	100	0,015	—62	—60
	0,0025	0,017	0,029	70		0,022	—58	
	0,00125	0,018	0,032	78		0,019	—93	
	0,000625	0,019	0,042	121		0,018	—104	
d (—) Alanin ..... (gehemmt mit Hippursäure von gleicher Konz.)	0,00125	0,017	0,030	76	88	0,024	—46	—50
	0,00125	0,020	0,040	100		0,029	—55	
d (—) Valin	0,00125	0,028	0,056	100		0,029	—96	
	0,000625	0,014	0,030	114		0,019	—69	
d (+) Phenylalanin...	0,00125	0,026	0,044	69	56	0,027	—94	—92
	0,00125	0,026	0,037	42		0,027	—91	

der Benzoesäure bei niedrigerer Substratkonzentration kräftiger geltend macht als bei höherer. Auch aus den im Folgenden geschilderten Versuchen mit anderen Aminosäuren ergibt sich, dass das Steigen der Substratkonzentration der Hemmungswirkung der Benzoesäure entgegenwirkt.

Die Versuche zeigen also, dass Benzoesäure die Desaminierung der l (+) Glutaminsäure hemmt, sowie *dass die Hemmung* bei niedrigeren Aminosäurekonzentrationen, die wahrscheinlich den unter physiologischen Verhältnissen vorkommenden Konzentrationen mehr entsprechen, *50 % übersteigt.*

Rechnet man, nach oben angeführter Motivierung, damit, dass die Variationsbreite der von der Benzoesäure auf die spontane Ammoniakbildung ausgeübten Hemmung 40—60 % beträgt, so wird die Hemmungswirkung bei den zwei niedrigsten l (+) Glutaminsäurekonzentrationen um ± 14 % resp. ± 25 % variieren. Im ersteren Fall dürfte also auch bei Berücksichtigung der Versuchsfehler die von der Benzoesäure auf die Desaminierung der l (+) Glutaminsäure ausgeübte Hemmung 50 % übersteigen, während sich dies im andern Fall weniger sicher sagen lässt. Wahrscheinlich verhält es sich aber doch auch in diesem Fall so; nur die Unsicherheit der Zahlen und hiermit die Variationsbreite ist wegen des hier geringeren Stoffwechsels gewachsen.

Im *Leberschnitt* ist die Stimulierung der Ammoniakbildung durch *l* (+) *Glutaminsäure* so unbedeutend, dass sie nicht mit Sicherheit als bestehend angenommen werden kann (Tabelle 9). Dies stimmt auch mit den Ergebnissen von Untersuchungen Anderer überein. So fanden Felix u. Naka (1940), dass l (+) Glutaminsäure im Rattenleber-Schnitt kaum und l (—) Asparaginsäure sehr wenig abgebaut wird. Schlussfolgerungen über die von Benzoesäure auf den Umsatz von l (+) Glutaminsäure ausgeübte Hemmung lassen sich also nicht ziehen.

Aus Tabelle 8 geht hervor, dass *l* (—) *Asparaginsäure* in 0,00125-mol Konzentration die Ammoniakbildung im *Nierenschnitt* ungefähr ebensostark stimuliert, wie es von l (+) Glutaminsäure gleicher Konzentration gilt, sowie dass die Hemmungswirkung der Benzoesäure ungefähr ebensostark ist.

Eine entsprechende Berechnung der Variation der von Benzoesäure auf die Desaminierung der l (—) Asparaginsäure ausgeübten Hemmungswirkung ergibt, wenn die Variation der von der Benzoesäure auf die spontane Ammoniakbildung ausgeübten Hemmung 40—60 % beträgt, die Werte ± 15 % resp. ± 10 % im einen und im andern Fall.

Die *Hemmungswirkung* der Benzoesäure auf die Desaminierung der l (—) Asparaginsäure im Nierenschnitt *dürfte* also wie bei der l (+) Glutaminsäure *50 %* erheblich *übersteigen.*

Im *Leberschnitt* übt *l* (—) *Asparaginsäure* im Gegensatz zu l (+) Glutaminsäure eine deutliche Stimulation der Ammoniakbildung aus (Tabelle 9), welch letztere jedoch von Benzoesäure nicht beeinflusst zu werden scheint (siehe auch Diagramm 8). Die in der Tabelle angegebene Hemmung liegt innerhalb der Fehlergrenzen der Versuche.

Es ist denkbar, dass eine *im Lebergewebe stattfindende Bildung von Hippursäure aus Benzoesäure* den Grund dafür darstellte, dass die Benzoesäure hier keine nachweisbare Giftwirkung auf die Ammoniakbildung ausübt. Aus den früherbesprochenen Versuchen (Tabelle 6) ging nämlich hervor, dass Hippursäure eine schwächere Hemmungswirkung auf die Ammoniakbildung im Nierengewebe ausübt als Benzoesäure, und nach Untersuchungen von Borsook u. Dubnoff (1940) synthetisiert die Rattenleber Hippursäure rascher als die Rattenniere. Indessen übt auch die Hippursäure eine deutliche Hemmungswirkung auf die spontane Ammoniakbildung im Nierengewebe aus, was dagegen spricht, dass eine im Lebergewebe vorsichgehende Synthese von Hippursäure aus Benzoesäure das Fehlen einer nachweisbaren Giftwirkung seitens der Benzoesäure auf die Ammoniakbildung im Lebergewebe genügend erklären könne. Hierauf soll weiter unten noch zurückgekommen werden.

Sowohl *l* (—) *Leucin* als auch *l* (—) *Prolin* in 0,00125-mol Konzentration stimulieren die Ammoniakbildung im *Nierenschnitt* recht wenig (Tabelle 8). Bei dieser Aminosäurekonzentration trat bei den vorhergehenden Versuchen die Hemmungswirkung der Benzoesäure besonders klar zutage, aber da es sich bei den in Rede stehenden Versuchen um kleine Werte handelt, ist es schwierig, die Stärke der vorliegenden Hemmung danach zu beurteilen. Es dürfte jedoch aus den Versuchen hervorgehen, dass die *Benzoesäure* in gewissem Umfang die *Ammoniakbildung* von l (—) Leucin und l (—) Prolin im Nierenschnitt *hemmt.*

Im *Leberschnitt* (Tabelle 9) stimulieren *l* (—) *Leucin* und *l* (—) *Prolin* die Ammoniakbildung in ungefähr gleichem Ausmasse wie l (—) Asparaginsäure, aber ebenso wie es bei dieser Säure der Fall war, liegen auch hier keine sicheren Anhaltspunkte dafür vor, dass die Ammoniakbildung durch Benzoesäure beeinflusst werde.

Aus den Versuchen scheint also hervorzugehen, dass *Benzoesäure auf die Desaminierung der untersuchten l-Aminosäuren im Nierenschnitt eine Hemmung ausübt.* Hinsichtlich der l (+) Glutamin-

säure und l (—) Asparaginsäure scheint diese Hemmung diejenige zu übertreffen, die Benzoesäure von gleicher Konzentration auf die spontane Ammoniakbildung im Nierenschnitt ausübt, während wegen der Unvollkommenheit der Versuchsmethode betreffs l (—) Leucin und l (—) Prolin nichts Sicheres hierüber ausgesagt werden kann. Da ausserdem nur einige Repräsentanten der Aminosäuren untersucht wurden, können keine sicheren generellen Schlussfolgerungen gezogen werden, aber die Versuche sprechen doch dafür, dass *l-Aminosäuren* in ihrer Eigenschaft als Ammoniakspender im Nierengewebe *besonders empfindlich gegen die Giftwirkung der Benzoesäure* sind.

Dagegen konnte bei diesen Versuchen eine Hemmung der Desaminierung von l-Aminosäuren im Lebergewebe ebensowenig nachgewiesen werden wie eine Hemmung der spontanen Ammoniakbildung in eben diesem Gewebe nachweisbar war.

Der Einfluss der Benzoesäure auf die Desaminierung der d-Aminosäuren.

Es wurden bereits (Seite 14 ff.) mehrere Umstände besprochen, die dafür sprechen, dass auch d-Formen beim Aminosäurestoffwechsel im Organismus eine Rolle spielen können. Aus diesem Grunde wurde nun auch untersucht, welchen Einfluss die Benzoesäure auf die Desaminierung von d-Aminosäuren ausübe.

Die früher referierten Untersuchungen von KLEIN u. KAMIN (1941) handelten allerdings von der *Hemmungswirkung der Benzoesäure* gerade *auf* das *d-Aminosäureoxydasenzym*. Die Versuche waren mit gereinigtem d-Aminosäureoxydasenzym oder mit Ratten-Nieren- und Leber-Schnitten ausgeführt worden. Die Verfasser haben aber bei ihren Versuchen nur den Sauerstoffverbrauch und nicht auch die Ammoniakbildung registriert. Dies dürfte genügen bei Versuchen mit gereinigtem hochspezifischem Enzym, aber bei Schnittversuchen, bei denen viele andere funktionstaugliche Enzyme vorhanden sind und viele natürliche Substrate vorkommen, reicht dies nicht aus, um Schlussfolgerungen über die Hemmungswirkung der Benzoesäure auf den Abbau gerade der zugesetzten Aminosäuren zu erlauben. Die Versuchsergebnisse, soweit sie auf Schnittversuche gegründet sind, dürften daher diskutabel bleiben; nach dem Bericht über meine eigenen Versuche werde ich darauf zurückkommen.

Als Repräsentanten für die d-Aminosäuren wurden bei den Untersuchungen *d(—)Alanin, d(—)Valin und d(+)Phenylalanin* verwendet. Die Versuche wurden nicht mit den reinen d-Isomeren, sondern mit den dl-Formen angestellt, da erstere nicht beigeschafft werden konnten. Dies dürfte jedoch keine grössere Rolle spielen, da die l-Formen dieser Aminosäuren in In-vitro-Versuchen mit Nierengewebe im

Vergleich zu den d-Formen in ausserordentlich geringem Masse abgebaut werden (KREBS 1933 a). Um die Resultate besser zu sichern, wurden jedoch Extraktversuche mit den gleichen Aminosäuren angestellt. Hierbei wird nämlich die l-Komponente, wie schon auf Seite 18 erwähnt, nicht abgebaut, da ihr Enzym schon vorher, während der Extraktbereitung, zerstört wird.

Aus Tabelle 8 geht hervor, dass d(—)Alanin bei Versuchen mit *Nierenschnitt* die Ammoniakbildung kräftig stimuliert. d(—)Valin und d(+)Phenylalanin stimulieren nicht ebensokräftig, aber doch noch genügend, um zuverlässige Schlussfolgerungen zuzulassen. Aus den Versuchen geht hervor, dass Benzoesäure bei einer angenommenen 50 %-igen Hemmung auf die spontane Ammoniakbildung, die Desaminierung aller drei Aminosäuren fast total hemmt.

Eine Variation der Hemmung der spontanen Ammoniakbildung zwischen 40 und 60 % beeinflusst die Hemmungswirkung der Benzoesäure auf die Desaminierung von d (—) Alanin von 0,000625-mol Konzentration um ± 8 resp. ± 14 %, von d (—) Valin von 0,00125-mol resp. 0,000625-mol Konzentration um ± 14 resp. ± 21 % und von d (+) Phenylalanin von 0,00125-mol Konzentration um ± 13 resp. ± 21 %.

Die *Benzoesäure dürfte* also *im Nierenschnitt die Desaminierung* aller *der* drei *d-Aminosäuren mehr hemmen als sie die spontane Ammoniakbildung* hemmt.

Die starke Hemmungswirkung der Benzoesäure zeigt sich auch bei *Nierenextrakt*versuchen. Aus Diagramm 16 wie aus Tabelle 8 geht ihre Wirkung auf d(—)Alanin hervor. Auf Diagramm 16 steht die spontane Ammoniakbildung auf dem gleichen Niveau wie die von d(—)Alanin stimulierte und zugleich durch Benzoesäure gehemmte. Dies zeigt also, dass die Hemmungswirkung der Benzoesäure auf die durch d(—)Alanin stimulierte Ammoniakbildung während der ganzen Versuchsdauer gleich intensiv ist. Die Werte in Tabelle 8, die sich ausschliesslich auf die Menge des nach 1¾ std Versuchsdauer gebildeten Ammoniaks beziehen, zeigen ebenfalls, dass diese Hemmung so gut wie total ist. Aus Tabelle 8 geht ferner hervor, dass Benzoesäure in Nierenextraktversuchen eine gleichstarke Hemmung auf die Desaminierung von d(—)Valin und d(+)Phenylalanin ausübt. *Die Versuche mit Nierenextrakt bestätigen* also *die Resultate der Versuche mit Nierenschnitten.*

Im *Leberschnitt* gelingt es, im Gegensatz zu dem, was für l-Aminosäuren gilt, durch d-Aminosäuren eine kräftige Stimulation der Ammoniakbildung zu bewirken (Tabelle 9). Dies zeigt sich an allen

drei untersuchten d-Aminosäuren. Dagegen lassen sich sichere Anhaltspunkte dafür, dass Benzoesäure die Desaminierung der d-Aminosäuren hemme, ebensowenig finden, wie es bei den l-Aminosäuren der Fall war.

Die Versuche mit *Leberextrakt*, im Gegensatz zu denen mit Leberschnitten, zeigen, dass *Benzoesäure die Desaminierung der zugesetzten d-Aminosäuren stark hemmt* (Tabelle 9). Dies spricht dafür, dass das Lebergewebe, solange seine Zellen, wie es bei den Schnittversuchen der Fall ist, weniger geschädigt sind, eine Möglichkeit hat, die Hemmung der Benzoesäure auf den Aminosäureabbau zu entgiften. Diese Fähigkeit scheint aber durch die Behandlung, der die Zellen bei der Extraktbereitung ausgesetzt werden, zu verschwinden, weshalb sich dann die Hemmungswirkung der Benzoesäure geltend machen kann. Wie bereits gesagt, liegt es nahe, zu vermuten, dass *die Entgiftung auf einer Bildung von Hippursäure* aus Benzoesäure *beruhe.* Auch die Bildung von Hippursäure setzt das Vorhandensein intakter Zellen voraus und findet bei Ratten, wie gesagt, in der Leber rascher als in der Niere statt, wie BORSOOK u. DUBNOFF (1940) gezeigt haben. Diese Forscher haben die Angabe von WAELSCH u. BUSZTIN (1937), dass Hippursäuresynthese auch bei Extraktversuchen stattfinden könne, nicht verifizieren können.

Es zeigt sich aber, dass *Hippursäure bei Leberextraktversuchen* zwar anscheinend eine etwas schwächere Hemmungswirkung auf die Desaminierung von d(—)Alanin ausübt als die Benzoesäure (Tabelle 9), was also darauf deuten könnte, dass Hippursäure auch im Lebergewebe weniger giftig wäre als Benzoesäure. Anderseits scheint sie aber auch hier nicht indifferent zu sein, weshalb das eben Gesagte keine genügende Erklärung dafür bieten dürfte, dass die Benzoesäure die Ammoniakbildung im Lebergewebe nicht hemmt. Hierzu kommt, dass nicht bloss Benzoesäure, sondern auch die meisten untersuchten Phenylderivate (Tabelle 6 und 7) im Gegensatz zu dem, was für das Nierengewebe gilt, eine wesentlich geringere oder garkeine Hemmungswirkung auf die spontane Ammoniakbildung im Lebergewebe ausübten. Dies gilt auch für Stoffe, die nicht in gleichem Umfang wie Benzoesäure mit Glykokoll hippursäureähnliche Verbindungen eingehen dürften, z. B. für die orthosubstituierten Benzoesäuren (QUICK 1932). Es scheint also ein ganz oder teilweise anderer Mechanismus zu sein, der die Giftwirkung von Benzoesäure und nahverwandten Stoffen im Lebergewebe aufhebt.

Angeregt durch Untersuchungen von EKMAN (1942) über die *Entgiftung zyklischer Verbindungen durch Ascorbinsäure* wurden einige Versuche angestellt, um zu erfahren, ob die Hemmungswirkung der Benzoesäure im Nierengewebe durch die Anwesenheit von Ascorbinsäure beeinflusst wird.

Die Versuche wurden mit Nierenschnitten angestellt, wobei der Stoffwechsel, um vor allem für die Ammoniakbildung grössere Werte und hierdurch zuverlässigere Resultate zu bekommen, durch l (+) Glutaminsäure stimuliert worden war. Die Konzentrationen von Benzoesäure und Ascorbinsäure wurden variiert, jedoch in einem ständigen gegenseitigen Verhältnis von 1 zu 8. Bei dieser Proportion erreicht EKMAN bei In-vitro-Versuchen mit Salicylsäure eine Umwandlung von ca. 65 % dieser Säure, während bei grösserer Ascorbinsäuremenge nur eine unbedeutende Erhöhung der Umwandlung von Salicylsäure erhalten wird.

TABELLE 10.

Der Einfluss der Benzoesäure auf den Sauerstoffverbrauch und die Ammoniakbildung im Nierenschnitt bei und ohne Anwesenheit von Ascorbinsäure. Stoffwechsel mit l (+) Glutaminsäure von 0,0025 mol Konc. stimuliert.

Ascorbinsäure-konc. Mol/l	Benzoesäure-konc. Mol/l	Sauerstoffverbrauch		Ammoniakbildung	
		Hemmung in %		Hemmung in %	
		ohne Ascorbinsäure	mit Ascorbinsäure	ohne Ascorbinsäure	mit Ascorbinsäure
0,1	0,0125	32	16	46	40
0,05	0,00625	27	5	42	42
0,025	0,00312	2	12	32	36
0,0125	0,00156	3	4	27	20

Aus der Tabelle ergibt sich anscheinend, dass die Hemmung des Sauerstoffverbrauchs bei Anwesenheit von Ascorbinsäure weniger ausgesprochen ist als ohne sie. Die Hemmung der Ammoniakbildung dagegen scheint in beiden Fällen die gleiche zu sein. Es ist somit denkbar, dass die Verbrennung der Ascorbinsäure die durch die Benzoesäure hervorgerufene Hemmung anderer Oxydationsprozesse maskiere, weswegen der Sauerstoffverbrauch bei Versuchen m i t Ascorbinsäure nicht so stark reduziert würde wie bei Versuchen o h n e Anwesenheit dieser Säure.

Aus der Tabelle ergibt sich auch, dass bei Annahme einer durch die Einwirkung von Ascorbinsäure hervorgerufenen Reduzierung

der Benzoesäure um 60—70 % die übrige Menge Benzoesäure doch gross genug ist, um eine Hemmung der Ammoniakbildung zu bewirken, die sich ihrer Stärke nach nicht deutlich von derjenigen unterscheidet, die durch die unverändert gebliebene Benzoesäuremenge in den entsprechenden Parallelversuchen verursacht wird, weshalb die Versuche nicht ausschliessen, dass eine Umwandlung von Benzoesäure durch Einwirkung der Ascorbinsäure vorliegt.

Aus den Untersuchungen mit d-Aminosäuren scheint also hervorzugehen, dass *Benzoesäure*, ebenso wie im Falle der l-Aminosäuren, eine *stärkere Hemmung auf die Desaminierung der d-Aminosäuren* ausübt *als auf die spontane Ammoniakbildung im Nierengewebe.* Weiterhin ist es von Interesse, dass diese *Hemmungswirkung der Benzoesäure* offenbar unter gewissen Voraussetzungen *auch im Lebergewebe* in Erscheinung tritt, obgleich sie dort normal blockiert zu werden scheint.

KLEIN und KAMIN (1941) haben in der auf Seite 11 erwähnten Arbeit u. a. die Hemmungswirkung der Benzoesäure auf den Abbau von d (—) Alanin im Leberschnitt untersucht. Sie fanden dabei, dass die gesamte Mehrung des Sauerstoffverbrauchs über den Spontanwert hinaus, die durch die Verbrennung des d (—) Alanins verursacht wird, bei Anwesenheit von Benzoesäure unterblieb. Daraus schlossen sie, dass Benzoesäure die Verbrennung des d (—) Alanins im Leberschnitt hemme wie bei Extraktversuchen und bei Versuchen mit gereinigtem d-Aminosäureoxydaseenzym. Meine Versuche, die ja Ammoniakbestimmungen mitumfassten, haben indessen gezeigt, dass der Abbau des d (—) Alanins im Leberschnitt wahrscheinlich nicht gehemmt wird, wohl aber im Leberextrakt. In früher beschriebenen Versuchen (Tabelle 7) wurde auch gezeigt, dass Benzoesäure den spontanen Sauerstoffverbrauch im Leberschnitt hemmt, und dieser dürfte es auch sein, der in den Versuchen von KLEIN u. KAMIN gehemmt wird, nicht aber der Abbau des d (—) Alanins.

Der Einfluss der Benzoesäure auf andere ammoniakbildende Prozesse.

Versuche mit Nierenextrakt zeigen, dass weder Benzoesäure noch Mandelsäure eine Hemmung auf die spontane Ammoniakbildung ausüben, die im Extrakt vorsichgeht (Diagramme 12, 13, 15), dies im Gegensatz zu den Verhältnissen bei der spontanen Ammoniakbildung im Nierenschnitt. Auch im Leberextrakt übt Benzoesäure keine Hemmung auf die spontane Ammoniakbildung aus (Diagramm 17 und Tabelle 5); ferner konnte im Leberschnitt nicht mit Sicherheit eine solche Hemmung nachgewiesen werden (Diagramm 6 und Tabelle 7).

In Anbetracht dieser ungleichen Wirkung der Benzoesäure im

Nierenschnitt und im Nierenextrakt liegt die bereits auf Seite 48 geäusserte Vermutung nahe, *das im Nierenschnitt entstehende Ammoniak sei nicht ausschliesslich gleichen Ursprungs wie das im Nierenextrakt entstehende.* Dies wird auch wahrscheinlich, wenn man bedenkt, dass l-Aminosäuren in Extraktversuchen nicht abgebaut werden, und dass ein Abbau von d-Aminosäuren unter natürlichen Verhältnissen in normalem Gewebe nicht nachgewiesen ist. Vermutlich stammt also das Ammoniak, das in Extrakten entsteht, von anderen Spendern als Aminosäuren, während das Ammoniak im Schnitt teils von Aminosäuren, teils von den anderen Spendern stammt. Wahrscheinlich sind es diese letzteren, die bei Nierenschnittversuchen dazu beitragen, dass die von der Benzoesäure auf die Ammoniakbildung ausgeübte Hemmung auch bei sehr starker Konzentration des Stoffes (0,1 Mol/l; Tabelle 13) nie total wird, denn nach den Versuchen, die mit Nierenschnitten unter Zusatz verschiedener Aminosäuren angestellt wurden, dürfte Benzoesäure von so starker Konzentration die Aminosäuredesaminierung ganz gehemmt haben. Bei der Ammoniakbildung bei Extraktversuchen sind somit wahrscheinlich andere Enzymsysteme als die aminosäuredesaminierenden wirksam, und diese anderen scheinen im Gegensatz zu letzteren von Benzoesäure nicht beeinflusst zu werden.

Die hier verwendete Versuchsmethode erlaubt es nicht, die betreffenden Ammoniakspender im Nieren- und Leber-Gewebe zu identifizieren. Dass es aber ausser den Aminosäuren noch eine Anzahl von Stoffen gibt, die in Nieren- und Leber-Gewebe Ammoniak bilden können, wurde auf Seite 20 ff. bereits besprochen.

Desaminierung von Muskeladenylsäure.

Unter den Stoffen, die Ammoniak abgeben können, scheint Muskeladenylsäure einer von denen zu sein, die an der Ammoniakbildung im Nierengewebe teilnehmen. Da die Hemmungswirkung der Benzoesäure im Nierengewebe deutlicher in Erscheinung tritt als im Lebergewebe und deshalb dort auch eingehender untersucht werden kann, wurde in erster Linie Muskeladenylsäure zum Gegenstand der Untersuchung gemacht.

Versuche mit *Muskeladenylsäure* zeigen, dass diese *sich* in verschiedener Hinsicht *verhält wie diejenigen Stoffe im Nieren- und Leber-Extrakt, die spontan Ammoniak abgeben.* Die Ähnlichkeit besteht teils in einer *gleichartigen Kinetik* der Ammoniakbildung,

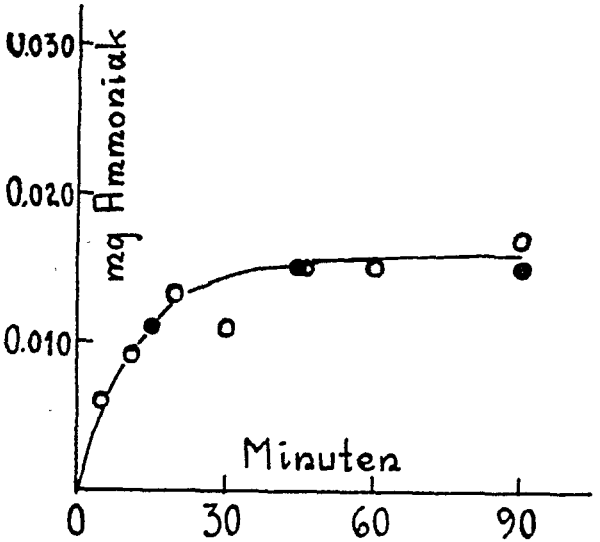


Diagramm 15. *Spontane Ammoniakbildung im Nierenextrakt* bei $1^1/_2$stündiger Versuchsdauer mit und ohne Benzoesäure in 0,025-mol Lösung. Der spontane Sauerstoffverbrauch während der Versuchszeit betrug ca. 40 mm³. Auf diesen übte Benzoesäure eine Hemmung von ca. 25 % aus.

Ohne Benzoesäure . . . . . ●——●
mit » . . . . . ○——○

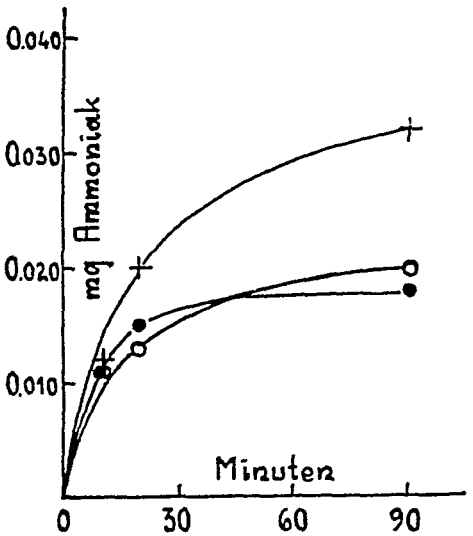


Diagramm 16. *Ammoniakbildung im Nierenextrakt* bei $1^1/_2$stündiger Versuchsdauer mit *dl-Alanin* von 0,00125-mol Konzentration, mit und ohne Benzoesäure von 0,025-mol Konz. in der Versuchslösung. Die spontane Ammoniakbildung während des Versuches ist angegeben. Der Sauerstoffverbrauch während der Versuchszeit betrug bei Anwesenheit von Alanin und Abwesenheit von Benzoesäure ca. 70 mm³. Benzoesäure übte auf den Sauerstoffverbrauch eine Hemmung von ca. 35 % aus.

Spontane Ammoniakbildung . . . . . ●— ●
Ammoniakbildung mit Alanin . . . . . +——+
» » » und Benzoesäure . . ○——○

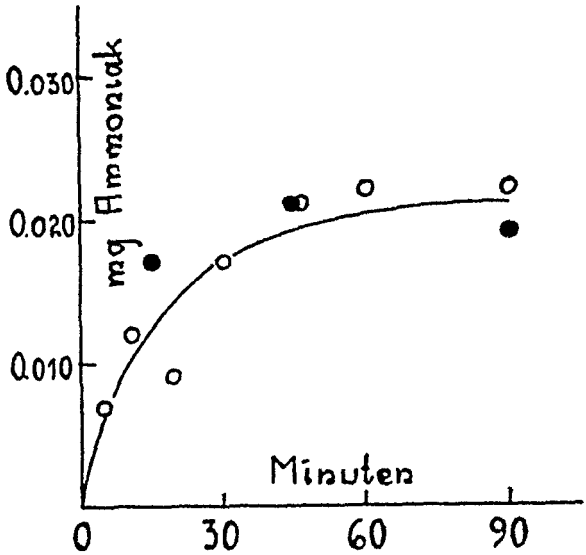


Diagramm 17. *Spontane Ammoniakbildung im Leberextrakt* bei $1^1/_2$stündiger Versuchsdauer mit und ohne Benzoesäure in 0,025-mol Lösung. Der spontane Sauerstoffverbrauch während der Versuchszeit betrug ca. 100 mm³. Auf diesen übte Benzoesäure eine Hemmung von ca. 5 % aus.

Ohne Benzoesäure . . . . . ●——●
mit » . . . . . ○——○

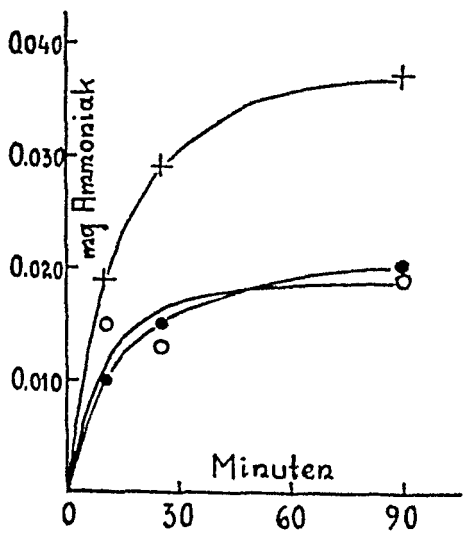


Diagramm 18. *Ammoniakbildung im Leberextrakt* bei $1^1/_2$stündiger Versuchszeit mit *dl-Alanin* von 0,00125-mol Konzentration in der Versuchslösung, mit und ohne Benzoesäure in 0,025-mol Lösung. Die spontane Ammoniakbildung während des Versuches ist angegeben. Der Sauerstoffverbrauch während der Versuchszeit betrug bei Anwesenheit von Alanin und Abwesenheit von Benzoesäure ca. 65 mm³. Letztere übte eine Hemmung von ca. 10 % auf den Sauerstoffverbrauch aus.

Spontane Ammoniakbildung . . . . . ●——●
Ammoniakbildung mit Alanin . . . . . +——+
» » » und Benzoesäure . . ○——○

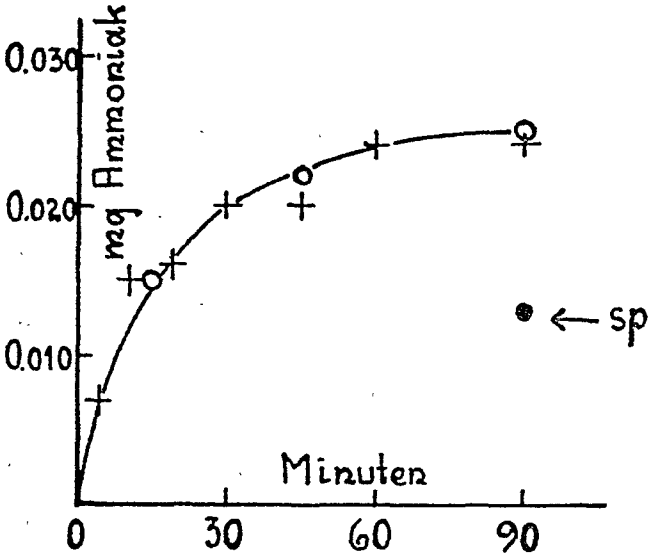


Diagramm 19. *Ammoniakbildung im Nierenextrakt* bei $1^1/_2$stündiger Versuchsdauer mit *Muskeladenylsäure* von 0,000312-mol Konzentration in der Versuchslösung, mit und ohne Benzoesäure in 0,025-mol Lösung. Das Mass der spontanen Ammoniakbildung am Ende der Versuchszeit ist angegeben. Der Sauerstoffverbrauch mit Muskeladenylsäure, aber ohne Benzoesäure, betrug bloss ca. 25 mm³ während der Versuchszeit, weshalb hierüber keine Schlussfolgerungen gezogen werden können.

Ohne Benzoesäure . . . . . +——+
mit » . . . . . O——O

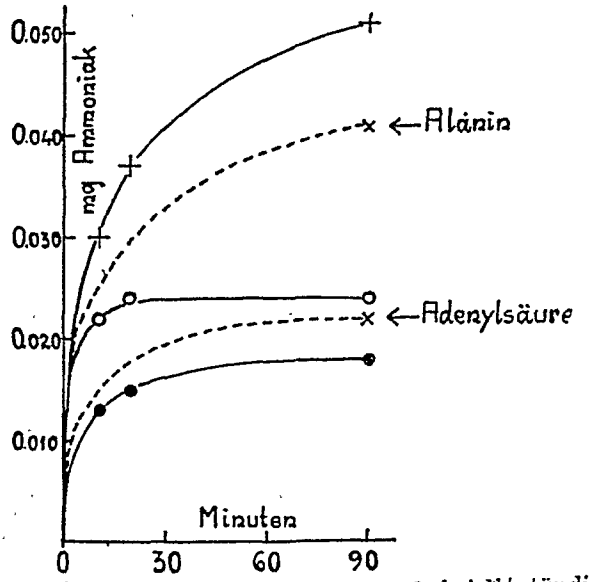


Diagramm 20. *Ammoniakbildung im Nierenextrakt* bei $1^1/_2$stündiger Versuchszeit mit *Muskeladenylsäure* von 0,000312-mol Konzentration *sowie dl-Alanin* von 0,0025-mol Konzentration in der Versuchslösung, mit und ohne Benzoesäure in 0,025-mol Lösung. Das Mass der Ammoniakbildung am Ende der Versuchszeit bei Anwesenheit von Muskeladenylsäure resp. Alanin je ohne Benzoesäure ist angegeben. Der Sauerstoffverbrauch während der Versuchszeit betrug mit Muskeladenylsäure und Alanin, aber ohne Benzoesäure nur ca. 25 mm³, weshalb sich hier keine Schlüsse ziehen lassen.

Spontane Ammoniakbildung . . . . . ●——●

teils in dem *Vermögen*, hinsichtlich ihrer Ammoniakproduktion *der Hemmungswirkung der Benzoesäure zu widerstehen.*

Die spontane Ammoniakbildung scheint in Nieren- und Leber-Extrakt-Versuchen viel rascher vor sich zu gehen als in Schnittversuchen (Diagramme 2, 6, 15, 17). Ebenso rasch scheint Ammoniakbildung im Nierenextrakt stattzufinden, wenn dieses durch Muskeladenylsäure stimuliert wird (Diagramm 19); in dem dort reproduzierten Versuch wird die Muskeladenylsäure zu ca. 70 % abgebaut. Dagegen scheint die Ammoniakbildung etwas verzögert zu werden, wenn Nierenextrakt durch eine Aminosäure stimuliert wird (Diagramme 16 und 20). Einen noch mehr in die Länge gezogenen Verlauf bekommt die Ammoniakbildung in einem Versuch mit Nierenschnitt, der durch eine Aminosäure stimuliert wird (Diagramm 4). Die Versuche sprechen also dafür, dass man durch Stimulation von Nierenextrakt mit Muskeladenylsäure eine Pointierung des Typus der spontanen Ammoniakbildung im Nierenextrakt erhält, durch die Stimulation von Nierenschnitt mit einer Aminosäure jedoch eine entsprechende Pointierung des Charakteristischen an der Ammoniakbildung im Nierenschnitt. Die *Aminosäuren* dürften also *im Nierenschnitt, Muskeladenylsäure im Nierenextrakt charakteristische Ammoniakspender* sein.

Die andere Ähnlichkeit zwischen der spontanen Ammoniakbildung im Nieren- und Leber-Extrakt und der durch Muskeladenylsäure stimulierten Ammoniakbildung im Nieren- und Leber-Gewebe ist, dass beide von Benzoesäure nicht gehemmt werden (Diagramme 19, 20; Tabellen 11, 12).

Aus Diagramm 19 geht hervor, dass die von Muskeladenylsäure stimulierte Ammoniakbildung im Nierenextrakt von Benzoesäure nicht beeinflusst wird, während die Ammoniakbildung aus d(—)Alanin, das neben Muskeladenylsäure die Ammoniakbildung in einem andern Versuch stimuliert (Diagramm 20), gehemmt zu werden scheint. Aus Tabelle 11 ergibt sich die kräftige *Stimulierung der Ammoniakbildung in* sowohl *Nierenschnitt-* als *Nieren-Extrakt-*Versuchen *durch Muskeladensylsäure*, einer Ammoniakbildung, die *durch Benzoesäure nicht gehemmt* wird.

Es wirkt auf das Resultat nicht nennenswert ein, wenn die durch Benzoesäure ausgeübte Hemmung der spontanen Ammoniakbildung in den Versuchen mit Nierenschnitt nicht, wie in Tabelle 11 der Berechnung zu Grunde gelegt, 50 % beträgt, sondern an anderer Stelle zwischen 40 und 60 % liegt.

TABELLE 11.

Einwirkung von Benzoesäure in 0,025-mol Lösung auf die Ammoniakbildung aus Muskeladenylsäure, dl-Asparagin und dl-Asparaginsäure im Nierengewebe.

Gewebe und Substrat	Substrat-Konz. Mol/l	Ammoniakbildung in mg		Stimulierung in %	Mittelwert	Ammoniakbildung mit Substrat und Benzoesäure in mg	Hemmung (—) resp. Stimulierung (+) der Substratstimulierung durch Benzoesäure in %	Mittelwert
		spontan	mit Substrat					
1	2	3	4	5	6	7	8	9
Nierenschnitt								
Adenylsäure	0,01	0,024	0,070	192		0,070	+26	
	0,01	0,022	0,067	204	203	0,064	+18	+19
	0,01	0,021	0,066	214		0,061	+12	
	0,0025	0,018	0,070	289		0,065	+8	
	0,000625	0,023	0,041	78	81	0,031	+8	+12
	0,000625	0,019	0,035	84		0,028	+16	
Nierenextrakt								
Adenylsäure	0,000625	0,012	0,033	175	140	0,032	—5	—2
	0,000625	0,018	0,037	106		0,037	0	
Nierenschnitt								
Asparagin	0,01	0,014	0,044	214		0,026	—37	
Asparaginsäure	0,01	0,014	0,054	286		0,016	—78	
Asparagin	0,00125	0,020	0,026	30		0,017	+17	
	0,00125	0,029	0,036	24	33	0,024	+36	+18
	0,00125	0,018	0,026	44		0,017	0	
Asparaginsäure	0,00125	0,020	0,033	65		0,016	—54	
	0,00125	0,029	0,039	34	63	0,015	—95	—79
	0,00125	0,018	0,034	89		0,011	—88	
Nierenextrakt								
Asparagin	0,01	0,008	0,023	188		0,023	0	
Asparaginsäure	0,01	0,008	0,008	0		0,007	—	
Asparagin	0,0025	0,013	0,017	31		0,016	—25	
Asparaginsäure	0,0025	0,013	0,016	23		0,014	—67	
Asparagin	0,000625	0,009	0,012	33		0,012	0	
Asparaginsäure	0,000625	0,009	0,010	11		0,008	—	

TABELLE 12.

Einwirkung von Benzoesäure in 0,025-mol Lösung auf die Ammoniakbildung aus Muskel-adenylsäure, dl-Asparagin und dl-Asparaginsäure, Benzamid, Acetamid und l (—) Histidin im Lebergewebe.

Gewebe und Substrat	Substrat-Konz. Mol	Ammoniakbildung in mg spontan	Ammoniakbildung in mg mit Substrat	Stimulierung in %	Mittelwert	Ammoniakbildung mit Substrat und Benzoesäure in mg	Hemmung (—) resp. Stimulierung (+) der Substratstimulierung durch Benzoesäure in %	Mittelwert
1	2	3	4	5	6	7	8	9
Leberschnitt								
Adenylsäure	0,01	0,017	0,032	88	82	0,030	—13	—6
	0,01	0,022	0,050	77		0,022	0	
	0,0025	0,021	0,028	33		0,027	—14	
Leberextrakt								
Adenylsäure	0,01	0,021	0,042	100		0,044	—10	
	0,0025	0,023	0,033	14		0,032	—25	
Leberschnitt								
Asparagin	0,01	0,016	0,071	344		0,072	—2	
Asparaginsäure	0,01	0,016	0,026	62		0,026	0	
Asparagin	0,00125	0,024	0,055	129	138	0,059	+13	—12
	0,00125	0,019	0,047	147		0,050	—11	
Asparaginsäure	0,00125	0,024	0,051	29	33	0,029	—28	—28
	0,00125	0,019	0,026	37		0,024	—28	
Asparagin	0,000625	0,016	0,012	20		0,012	0	
Asparaginsäure	0,000625	0,010	0,010	0		0,012	—	
Leberextrakt								
Asparagin	0,0025	0,028	0,079	182		0,076	—6	
Asparaginsäure	0,0025	0,028	0,032	14		0,031	—25	
Asparagin	0,00125	0,020	0,038	90		0,036	—11	
Asparaginsäure	0,00125	0,020	0,022	10		0,023	—50	
Benzamid	0,0025	0,029	0,058	100	114	0,034	—83	—66
	0,0025	0,018	0,041	128		0,030	—48	
Benzamid (gehemmt mit Salicylsäure von gleicher Konz.)	0,0025	0,025	0,047	88		0,037	—45	
Acetamid	0,0025	0,036	0,047	30	32	0,040	—64	—57
	0,0025	0,036	0,048	33		0,042	—50	
l (—) Histidin	0,0025	0,038	0,176	363	270	0,163	—9	—3
	0,0025	0,038	0,105	176		0,107	+3	

Im *Leberschnitt und Leberextrakt* wird die Ammoniakbildung durch Muskeladenylsäure nicht so kräftig stimuliert (Tabelle 12). Auch bei diesen Versuchen übt *Benzoesäure keine Hemmung* auf die Ammoniakbildung aus. Es ist von besonderem Interesse, dass die Benzoesäure in Leberextrakt-Versuchen keine Hemmung auf die Ammoniakbildung aus Muskeladenylsäure ausübt; denn in Versuchen mit d-Aminosäuren zeigte sich, dass Benzoesäure bei entsprechenden Versuchen die Fähigkeit hatte, die Ammoniakbildung zu hemmen.

Desamidierung von Asparagin.

Dass Asparagin und Glutamin als Ammoniakspender im Nieren- und Leber-Gewebe auftreten können, wurde bereits auf Seite 20 besprochen.

Die Versuche wurden mit Asparagin gemacht. Die Ergebnisse sind schwerer zu deuten als die der früheren Versuche, da es denkbar ist, dass Ammoniak sowohl aus der Amino- als auch aus der Amidgruppe gebildet wurde. Um ein sichereres Resultat zu erreichen, wurden deshalb in jeder Versuchsserie einzelne Versuche mit sowohl dl-Asparagin als auch dl-Asparaginsäure von gleicher Konzentration angestellt. Durch einen Vergleich der Ammoniakbildung aus den beiden Stoffen je für sich, ist es leichter, eine Vorstellung davon zu bekommen, woher das vom Asparagin abgegebene Ammoniak stammt, und wie die Benzoesäure auf das betreffende Enzymsystem einwirkt.

Die Ergebnisse finden sich in Tabelle 11 und 12. Es zeigt sich, dass Asparagin die Ammoniakbildung im *Nierenschnitt* recht kräftig stimuliert, dass aber Asparaginsäure sie anscheinend noch etwas kräftiger stimuliert. Benzoesäure hemmt die von stärker konzentriertem Asparagin stimulierte Ammoniakbildung, beeinflusst aber die Ammoniakbildung nicht sicher, wenn das Asparagin in schwächerer Konzentration vorliegt. Im Gegensatz hierzu wird Asparaginsäure in den untersuchten Konzentrationen in ihrer Ammoniakbildung kräftig gehemmt, was auch durch frühere Versuche schon gezeigt worden war (Seite 69).

Die Erklärung hierfür könnte darin zu sehen sein, dass das Asparagin bei stärkerer Konzentration in gewissem Umfang desaminiert wird, und dass dieser Prozess im Gegensatz zur Ammoniakbildung aus der Amidgruppe gegen Benzoesäure empfindlich sein dürfte. Für die Richtigkeit dieser Erklärung ist Voraussetzung, dass die

Amidgruppe des Asparagins seine Desaminierung in grossem Umfang hemmt. Dafür, dass dem wirklich so sei, spricht auch, dass das Asparagin eine geringere Menge Ammoniaks abgibt als Asparaginsäure von gleicher Konzentration, obgleich es sowohl eine Amid- als auch eine Amino-Gruppe enthält. Es würde also eine Art von Konkurrenzhemmung vorliegen.

Auch hier wirkt es nicht wesentlich auf die Frage der Richtigkeit der oben gezogenen Folgerungen ein, wenn bei den Nierenschnittversuchen die von Benzoesäure auf die spontane Ammoniakbildung ausgeübte Hemmung nicht, wie der Berechnung in Tabelle 11 zugrundegelegt ist, 50 %ig ist, sondern an irgend einer Stelle zwischen 40 und 60 % liegt.

Die Versuche mit *Nierenextrakt* scheinen zu verifizieren, dass das Nierengewebe Asparagin desamidieren kann, denn die Aminogruppe der dl-Asparaginsäure wird bei Extraktversuchen in sehr geringem Grade desaminiert, da die d-Komponente bei In-vitro-Versuchen überhaupt in sehr geringem Masse, und die l-Komponente bei Extraktversuchen nur wenig abgebaut wird. In Analogie hiezu ist es wahrscheinlich, dass bei Extraktversuchen auch die Aminogruppe des Asparagins nicht desaminiert wird. *Benzoesäure scheint, wie bei Nierenschnittversuchen, die Desamidierung des Asparagins nicht zu hemmen.*

In *Leberschnittversuchen* scheint, wie zu erwarten, weder die durch Asparagin noch die durch Asparaginsäure stimulierte Ammoniakbildung gehemmt zu werden. Auch bei *Leberextraktversuchen* wird die *von Asparagin herrührende Ammoniakbildung nicht gehemmt*, was, wie die entsprechende Erscheinung bei den Versuchen mit Muskeladenylsäure, von Interesse ist, da Benzoesäure die Desaminierung derjenigen Aminosäuren hemmt, die die Ammoniakbildung im Leberextrakt stimulieren.

Es wäre zu erwarten gewesen, dass die Benzoesäure die Desaminierung der d-Komponente der dl-Asparaginsäure gehemmt hätte, aber, wie oben gesagt, wird sie bei In-vitro-Versuchen in sehr geringem Masse abgebaut.

Desamidierung von Benzamid und Acetamid.

Im Hinblick auf die Fähigkeit des Organismus auch einfache Amide zu desamidieren (Seite 21) war es von Interesse, die Empfindlichkeit dieser Prozesse gegen Benzoesäure ebenfalls zu studieren. Die Versuche wurden nicht in Leberschnitten gemacht, da die Hemmungswirkung der Benzoesäure hier nicht in Erscheinung tritt,

sondern in Leberextrakt, wo Benzoesäure die Desaminierung der d-Aminosäuren stark beeinflusst. Als Substrat wurde Benzamid und Acetamid verwendet.

Die Resultate finden sich auf Tabelle 12. Sie zeigen, dass Benzamid die Ammoniakbildung kräftig mehrt, während Acetamid eine schwächere Wirkung ausübt. Die *Desamidierung des Benzamids* wird, wie die Desaminierung des d(—)Alanins in entsprechenden Versuchen (Tabelle 9), *von Benzoesäure und* auch von *Salicylsäure* ungefähr gleichstark *gehemmt*. In Anbetracht der sehr ähnlichen Konstitution der Benzoesäure und des Benzamids ist es denkbar, dass eine Konkurrenzhemmung der Enzymtätigkeit vorliegt. Da aber auch Salicylsäure die Desamidierung hemmt, und da *auch die Desamidierung von Acetamid durch Benzoesäure gehemmt* zu werden scheint, kann man vermuten, dass die Hemmungswirkung der Benzoesäure von mehr genereller Art sei. Dies ist bemerkenswert, weil die Desamidierung des Aminosäureamids Asparagin durch Benzoesäure anscheinend nicht beeinflusst wurde.

Ammoniakbildung aus der Imidazolgruppe des Histidins.

Im vorhergehenden (Seite 22) wurden Gründe angegeben für eine Untersuchung des Einflusses der Benzoesäure auch auf die Ammoniakbildung, die der Imidazolgruppe des l (—) Histidins entstammt, und die durch Histidase vermittelt wird.

Die Versuche, die mit Leberextrakt gemacht wurden, zeigen (Tabelle 12), dass eine sehr kräftige Stimulierung der Ammoniakbildung stattfindet. Im einen Versuch scheint die Menge des gebildeten Ammoniaks ungefähr derjenigen zu entsprechen, die aus einem Äquivalent Stickstoff im Imidazolring gebildet werden kann. *Benzoesäure* scheint indessen die *Ammoniakbildung nicht zu beeinflussen.*

Zusammenfassung der Ergebnisse.

Die Ergebnisse der Untersuchungen über den Einfluss der Benzoesäure auf verschiedene ammoniakbildende Prozesse zusammenfassend lässt sich sagen, dass die verschiedenen Enzymsysteme offenbar ungleich empfindlich gegen Benzoesäure sind. Während Adenylsäuredesaminase, Asparaginase und Histidase anscheinend nicht beeinflusst werden, werden die einfachen Amidasen, die Benzamid und Acetamid abbauen, ebenso wie die aminosäureabbauenden Enzyme gehemmt.

Auf Seite 64 wurde die Frage erörtert, ob die ungefähr 50 %ige Hemmung der spontanen Ammoniakbildung, die bei Versuchen mit Nierenschnitten durch Benzoesäure von 0,025-mol Konzentration hervorgerufen wird, sich aus einer ungefähr gleichstarken Hemmung sämtlicher ammoniakbildenden Prozesse ergebe, oder aber daraus, dass gewisse Prozesse in stärkerem, andere in geringerem Grade gehemmt würden, wobei der Mittelwert 50 % betrüge. Die vorliegenden Untersuchungen zeigen, dass letzteres der Fall sein dürfte. Soweit es sich nach eben diesen Untersuchungen beurteilen lässt, dürfte hinter der durch die Benzoesäure hervorgerufenen Hemmung der spontanen Ammoniakbildung im Nierenschnitt die starke Hemmung der Aminosäuredesaminierung liegen. Freilich geht aus den Untersuchungen hervor, dass Benzoesäure im Lebergewebe auch die Ammoniakbildung aus Benzamid und Acetamid hemmt, aber deren Rolle und die Rolle mit ihnen verwandter Stoffe als Ammoniakspender dürften nicht von so wesentlicher Bedeutung sein.

Der Einfluss der Benzoesäure auf die spontane Ammoniakbildung in verschiedenen Geweben.

Aus den bisherigen Untersuchungen ging hervor, dass Grund zu der Annahme besteht, Benzoesäure in einer Konzentration von 0,025 Mol/l blockiere die Aminosäuredesaminierung im Nierenschnitt ziemlich spezifisch, während andere ammoniakbildende Prozesse nicht beeinflusst würden. Durch Benzoesäure von stärkerer Konzentration wird die spontane Ammoniakbildung im Nierenschnitt nicht merkbar stärker beeinflusst, was zeigt, dass die Benzoesäure bei dieser Konzentration die gegen sie empfindlichen Prozesse nahezu vollständig gehemmt hat. Erst bei sehr hoher Konzentration, bei der unter anderm eine unspezifische Salzwirkung eine bedeutende Rolle spielen kann, tritt eine wesentliche Steigerung der Hemmungswirkung der Benzoesäure auf. Dies ist in Tabelle 13 dargestellt.

Wenn man also damit rechnet, dass *Benzoesäure* in einer Konzentration von 0,025 Mol/l *die Aminosäuredesaminierung spezifisch blockiert*, so würde man durch Zusetzen von Benzoesäure in dieser Konzentration *bei Versuchen mit verschiedenen Geweben* einen Begriff davon bekommen können, ein wie grosser Teil der spontanen Ammoniakbildung in den betreffenden Geweben Aminosäuren ent-

TABELLE 13.

Die Stärke der spontanen Ammoniakbildung in einem Versuch mit Nierenschnitt unter Einwirkung von Benzoesäure verschiedener Konzentrationen.

Benzoesäurekonz. (Mol/l)	Ammoniakbildung in mg	Hemmung der Ammoniakbildung in %
0,2	0,006	70
0,1	0,011	45
0,025	0,012	40
0,00312	0,015	25
0,000	0,020	—

stammt. Es wurden einige Untersuchungen in dieser Richtung angestellt; die Berechnungen sollen weiter unten diskutiert werden.

Das Ergebnis der vorher mitgeteilten Versuche mit *Nierenschnitten* (Tabelle 6), aus denen hervorgeht, dass Benzoesäure in einer Konzentration von 0,025 Mol/l die spontane Ammoniakbildung um ca. 50 % hemmt, spricht also dafür, dass ungefähr *die Hälfte des im Nierenschnitt spontan gebildeten Ammoniaks* von der Desaminierung *von Aminosäuren herrührt.*

Bei den zuvor mitgeteilten Versuchen mit *Leberschnitten* (Tabelle 7) konnte für Benzoesäure in der Konzentration von 0,025 Mol/l eine Hemmungswirkung nicht sicher nachgewiesen werden, was wahrscheinlich darauf beruht, dass die Benzoesäure entgiftet wird. Es ist daher nicht möglich, nach dieser Methode einen Begriff davon zu bekommen, wie gross die Rolle ist, die die Aminosäuren als Ammoniakspender im Lebergewebe spielen.

Auch die im *Milzgewebe* und im *Muskelgewebe* herrschenden Verhältnisse wurden studiert.

Das Milzgewebe wurde mit Hilfe von Schnittversuchen untersucht; die Methode war die gleiche wie bei den Versuchen mit Nieren - und Leber-Schnitten.

Das Muskelgewebe wurde nicht in Schnittversuchen untersucht, da es zu grosse Schwierigkeiten bot, geeignete Muskelschnitte herzustellen. Statt dessen wurde Muskelbrei verwendet, der durch Feinschneiden von Extremitätenmuskulatur bereitet wurde. Es wurden entweder 500 oder 700 mg Muskelbrei in jedem Warburggefäss verwendet. Im übrigen wurden diese Versuche auf gleiche Art wie die Schnittversuche ausgeführt.

Die Ergebnisse der Versuche mit Milz- und Muskel-Gewebe sind in Tabelle 14 wiedergegeben.

TABELLE 14.

Der Einfluss der Benzoesäure auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung im Milzgewebe und im Muskelgewebe.

Gewebe Benzoesäurekonc.	Sauerstoffverbrauch in mm³		Hemmung in %	Ammoniakbildung in mg		Hemmung (—) oder Stimulierung (+) in %
	ohne Benzoesäure	mit Benzoesäure		ohne Benzoesäure	mit Benzoesäure	
Milzschnitt (100 mg)						
0,1 Mol/l	161	72	55	0,005	0,002	—60
0,025 Mol/l	161	121	25	0,005	0,005	0
0,025 Mol/l	156	118	24	0,010	0,008	—20
Muskelbrei (500 mg)						
0,1 Mol/l	52	32	38	0,027	0,026	—4
0,025 Mol/l	52	42	19	0,027	0,027	0
Muskelbrei (700 mg)						
0,1 Mol/l	86	53	38	0,032	0,036	+12
0,025 Mol/l	86	71	17	0,032	0,030	—6

Aus Tabelle 14 ergibt sich, dass der spontane Sauerstoffverbrauch in sowohl Milz- als auch Muskel-Gewebe durch Benzoesäure in 0,025-mol Lösung gehemmt wird. Die spontane Ammoniakbildung ist im Milzgewebe gering, weshalb die Schlussfolgerungen wenig zuverlässig werden; es scheint aber doch, als hemme Benzoesäure von betreffender Konzentration sie weder im Milz- noch im Muskel-Gewebe.

Dies würde also dafür sprechen, dass *die spontane Ammoniakbildung in diesen Geweben, nicht von Aminosäuren*, sondern von andern Ammoniakdonatoren *herstamme*.

Diese Vermutung wird auch durch andere Untersuchungen gestützt. So hat KREBS (1933 a) gezeigt, dass dl-Alanin als Substrat bei Versuchen mit Milz- und Muskel-Gewebe von Ratten die spontane Ammoniakbildung nicht deutlich stimuliert. Das Gleiche ist der Fall bei l (—) Asparaginsäure in Versuchen mit Muskulatur (KREBS, 1935 a). Aus Tabelle 15 lässt sich auch ersehen, dass l (+) Glutaminsäure bei Versuchen mit Milz- und Muskel-Gewebe die Ammoniakbildung nicht zu stimulieren scheint, während bei Versuchen mit stärker konzentriertem Muskelbrei der Sauerstoffverbrauch etwas stimuliert wird.

TABELLE 15.

Der Einfluss der l (+) Glutaminsäure in der Konzentration von 0,0025 Mol/l auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung in Milz- und Muskel-Gewebe.

Gewebe	Sauerstoffverbrauch in mm³		Stimulierung in %	Ammoniakbildung in mg		Hemmung (—) oder Stimulierung (+) in %
	ohne Glutaminsäure	mit Glutaminsäure		ohne Glutaminsäure	mit Glutaminsäure	
Milzschnitt						
(100 mg)	156	164	5	0,011	0,012	+9
Muskelbrei						
(200 mg)	33	34	3	0,019	0,019	0
(500 mg)	63	72	14	0,033	0,029	—12
(700 mg)	111	139	25	0,038	0,041	+8

Es scheint also, als fehle dem Milz- und dem Muskel-Gewebe die Fähigkeit, Aminosäuren zu desaminieren. Ausserdem spricht, was auf Seite 22 bereits hervorgehoben wurde, das reichliche Vorkommen von Adenosindesaminase im Milzgewebe und von Adenylsäuredesaminase im Muskelgewebe (CONWAY u. COOKE 1939) dafür, dass die genannten Purinverbindungen in den betreffenden Geweben als wichtige Ammoniakspender wirken.

Die Annahme, dass das Fehlen einer nachweisbaren Hemmungswirkung auf die spontane Ammoniakbildung im Milz- und Muskel-Gewebe darauf beruhe, dass diese Ammoniakbildung nicht von einer Aminosäuredesaminierung herrührt, wird also auch durch das eben Gesagte gestützt.

Der Einfluss der Benzoesäure, Mandelsäure und Salicylsäure auf die Aminosäuredesaminierung bei Bacterium coli.

Die Versuche wurden nach der auf Seite 29 beschriebenen Methode ausgeführt. Es diente hierzu ein pathogener Stamm von Bacterium coli, von einem Patienten mit Colicystopyelitis. Als *Substrat* für die Bakterien wurde in den Warburggefässen *dl-Alanin, l (+) Alanin* und *l (+) Glutaminsäure* verwendet, und zwar in der Konzentration 0,00125 Mol/l, die auch für die Versuche mit tierischen Geweben

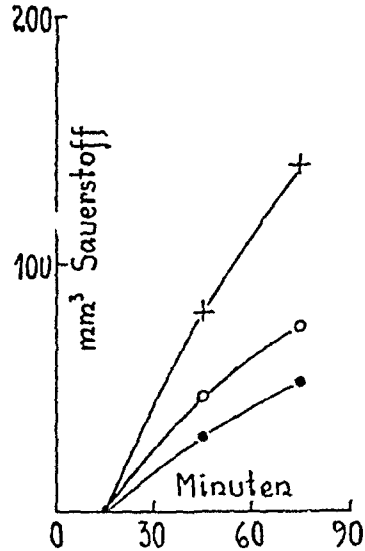


Diagramm 21. Grösse des *Sauerstoffverbrauchs* bei *Bacterium coli* bei 1¼stündiger Versuchszeit.

Spontaner Sauerstoffverbrauch . . . . . ●——●
Sauerstoffverbrauch mit 0,00125-mol dl-Alaninlösung . . . . . +——+
» » » »
und 0,1-mol Benzoesäurelösung . . . . ○——○

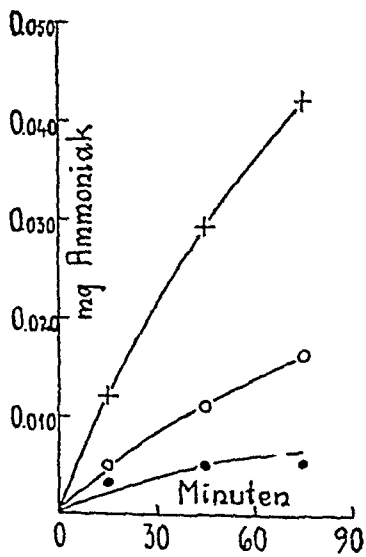


Diagramm 22. *Ammoniakbildung* bei dem auf Diagramm 21 dargestellten Versuch.

Spontane Ammoniakbildung . . . . . ●——●
Ammoniakbildung mit 0,00125-mol dl-Alaninlösung . . . . . +——+
» » » »
und 0,1-mol Benzoesäurelösung . . . . ○——○

TABELLE 16.

Einwirkung von Benzoesäure, Mandelsäure und Salicylsäure auf den spontanen Sauerstoffverbrauch und die spontane Ammoniakbildung bei Bact. coli.

Stoff (Konz. Mol/l in der Versuchsmischung)	Sauerstoffverbrauch in mm³		Hemmung (—) resp. Stimulierung (+) in %	Ammoniakbildung in mg		Hemmung in %
	spontan	mit resp. Stoff		spontan	mit resp. Stoff	
Benzoesäure 0,025	32	30	—6	0,007	0,007	0
Mandelsäure 0,025	32	31	—3	0,007	0,006	14
Salicylsäure 0,025	32	33	+3	0,007	0,007	0
Benzoesäure 0,1	60	62	+3	0,010	0,010	0
Mandelsäure 0,1	60	61	+2	0,010	0,010	0
Salicylsäure 0,1	60	55	—8	0,010	0,009	10

gewöhnlich gewählt wurde. Aminosäuren von sowohl d-als l-Form wurden untersucht, um die Frage zu studieren, ob eine Ungleichheit in der Wirkung der untersuchten Hemmungsstoffe auf sie vorliege. *Benzoesäure*, *Mandelsäure* und *Salicylsäure* wurden auf die Wirkung untersucht, die sie bei Anwesenheit der Substrate auf den *Sauerstoffverbrauch* und die *Ammoniakbildung* der Bakterien ausüben. Die Stoffe wurden teils in der bei den Versuchen mit tierischem Gewebe standardisierten Konzentration von 0,025 Mol/l, teils in einer Konzentration von 0,1 Mol/l untersucht, da bei der schwächeren Konzentration die Wirkung nicht immer mit genügender Deutlichkeit hervortrat.

In den Versuchen kam bei den Bakterien stets ein gewisser *spontaner Stoffwechsel* vor, was wahrscheinlich darauf beruhte, dass die Bakterien von dem Substrat, in dem sie gewachsen waren, nicht völlig befreit werden konnten. Der spontane Stoffwechsel betrug im allgemeinen ungefähr 1/3 resp. 1/5 des bei den Versuchen mit Substratzusatz auftretenden totalen Sauerstoffverbrauchs resp. der totalen Ammoniakbildung (Diagramme 21 und 22).

Aus Tabelle 16 lässt sich ersehen, dass der spontane Stoffwechsel *durch die untersuchten Stoffe* in der Regel anscheinend *wenig beeinflusst wird.* Nur Salicylsäure in einer Konzentration von 0,1 Mol/l scheint eine leichte Hemmung auf sowohl den Sauerstoffverbrauch als auch die Ammoniakbildung auszüben, weshalb nur bei Versuchen mit dieser Säure eine Beeinflussung des spontanen Stoffwechsels in Betracht zu ziehen ist.

TABELLE 17.

Einwirkung von Benzoesäure, Mandelsäure und Salicylsäure auf den Sauerstoffverbrauch und die Ammoniakbildung bei Bact. coli in Anwesenheit von 0,00125 Mol/l dl-Alanin resp. l (+) Glutaminsäure.

Substrat Stoff (Konz. Mol/l in der Versuchsmischung)	Sauerstoffverbrauch in mm³ [1])		Hemmung (—) resp. Stimulierung (+) in %	Mittelwert	Ammoniakbildung in mg [1])		Hemmung (—) resp. Stimulierung (+) in %	Mittelwert
	mit Substrat	mit Substrat und resp. Stoff			mit Substrat	mit Substrat und resp. Stoff		
dl-Alanin:								
Benzoesäure 0,025 ..	48	31	—35	—32	0,020	0,013	—35	—34
	100	71	—29		0,031	0,021	—32	
Mandelsäure 0,025 ..	53	34	—36	—40	0,021	0,012	—43	—40
	68	38	—44		0,028	0,018	—36	
Salicylsäure 0,025 ..	78	37	—52	—54	0,028	0,007	—75	—75
	62	27	—56		0,028	0,007	—75	
Natriumchlorid 0,1	82	95	+16		0,028	0,031	+11	
Benzoesäure 0,1	87	23	—74		0,037	0,011	—70	
Mandelsäure 0,1	43	18	—58		0,016	0,007	—56	
Salicylsäure 0,1	76	0	—100		0,019	0,000	—100	
l (+) *Glutaminsäure:*								
Benzoesäure 0,025 ..	102	108	+6	+6	0,031	0,030	—3	—8
	112	120	+7		0,032	0,028	—12	
Mandelsäure 0,025 ..	100	114	+14	+6	0,020	0,032	+10	+2
	157	156	—1		0,030	0,037	—5	
Salicylsäure 0,025 ..	140	59	—58		0,030	0,013	—57	
Natriumchlorid 0,1	122	108	—11		0,030	0,028	—7	
Benzoesäure 0,1	116	78	—33		0,030	0,022	—27	
Mandelsäure 0,1	103	137	+33	+24	0,026	0,031	+19	+14
	100	116	+16		0,026	0,028	+8	
Salicylsäure 0,1	53	—6	—111	—110	0,013	0,004	—69	—78
	62	—6	—110		0,016	0,002	—88	

Die Ergebnisse der Versuche mit *dl-Alanin* gehen aus Tabelle 17 hervor. Es zeigt sich, dass sämtliche drei untersuchten Stoffe schon in der schwächeren Konzentration eine *deutliche Hemmung auf den Stoffwechsel* ausüben. Wie bei den Versuchen mit Nieren- und Leber-Gewebe scheint Salicylsäure die stärkste Wirkung zu haben. In der stärkeren Konzentration hemmt sie den Abbau des dl-Alanins total.

[1]) Nach Abzug der Werte f. d. spont. Stoffwechsel.

TABELLE 18.

Einwirkung von Benzoesäure, Mandelsäure und Salicylsäure in einer Konz. von 0,025 Mol/l auf den Sauerstoffverbrauch und die Ammoniakbildung bei Bact. coli in Anwesenheit von 0,00125 Mol/l dl-Alanin resp. l (+) Alanin.

Stoff	Substrat	Sauerstoffverbrauch in mm³ [1])		Hemmung in %	Ammoniakbildung in mg [1])		Hemmung in %
		mit Substrat	mit Substrat und resp. Stoff		mit Substrat	mit Substrat und resp. Stoff	
Benzoesäure	dl-Alanin	71	56	21	0,021	0,016	24
»	l (+) Alanin	65	50	23	0,020	0,015	25
Mandelsäure	dl-Alanin	60	35	42	0,022	0,016	27
»	l (+) Alanin	58	33	43	0,019	0,013	32
Salicylsäure	dl-Alanin	57	30	47	0,027	0,012	56
»	l (+) Alanin	55	32	42	0,023	0,009	61

Es wurden auch Vergleiche zwischen der Wirkung der drei Stoffe auf den Abbau von dl-Alanin resp. l (+) Alanin angestellt (Tabelle 18). Hierbei wurde im gleichen Versuch jeder Stoff hinsichtlich seiner Wirkung auf den Stoffwechsel von dl- und l (+) Alanin je für sich untersucht. Es zeigte sich, dass, im Gegensatz zu dem, was für Nieren- und Leber-Gewebe gilt, d (—) Alanin und l (+) Alanin von Bacterium coli offenbar gleich rasch abgebaut werden. Dies geht auch aus den Versuchen von STEPHENSON u. GALE (1937) hervor. Die drei untersuchten Stoffe scheinen auch jeder für sich eine *gleichstarke Hemmung auf die beiden Komponenten des dl-Alanins* auszuüben.

Aus Tabelle 17 geht weiterhin hervor, dass Benzoesäure und Mandelsäure in der schwächeren Konzentration im Gegensatz zu den Versuchen mit Alanin keine sichere Wirkung auf den Abbau von *l (+) Glutaminsäure* ausüben. Dagegen übt Salicylsäure eine ausgesprochene Hemmung aus. Werden die Stoffe in der stärkeren Konzentration untersucht, kommt jedoch eine *Hemmung durch Benzoesäure* heraus, und die Hemmungswirkung der *Salicylsäure* scheint eine totale zu werden. Dagegen lässt sich bei den untersuchten Konzentrationen *keine Hemmung durch Mandelsäure* nachweisen. Es liegt im Gegenteil eine leichte Stimulierung von

[1]) Nach Abzug der Werte f. d. spont. Stoffwechsel.

sowohl Sauerstoffverbrauch als auch Ammoniakbildung vor. Diese Tatsache ist schwer zu erklären. Es ist jedoch denkbar, dass die Steigerung des Sauerstoffverbrauchs auf einer in gewissem Ausmass stattfindenden Verbrennung der Mandelsäure beruht. Besonders schwierig ist es dabei, die Stimulierung der Ammoniakbildung zu erklären. Da die Steigerung der Ammoniakbildung geringer ist als die des Sauerstoffverbrauchs, beruht sie vielleicht auf Versuchsfehlern; jedenfalls kann man sich kaum vorstellen, auf welche Art Mandelsäure die Ammoniakbildung aus anderen Stoffen stimulieren sollte.

Die Versuche mit dl-Alanin und l (+) Glutaminsäure weisen also untereinander mehrere Verschiedenheiten auf. Diese beruhen wahrscheinlich nicht auf der verschiedenartigen sterischen Konfiguration der Stoffe, sondern auf ihrer verschiedenen Zusammensetzung; die Versuche mit Alanin haben nämlich gezeigt, dass die Konfiguration in diesem Fall die Wirkung der verschiedenen Gifte offenbar nicht beeinflusst.

Aus den Versuchen mit Colibakterien ergibt sich also, dass *Benzoesäure und nahverwandte Stoffe hier wie im Nieren- und Leber-Gewebe eine im grossen und ganzen gleichartige Hemmung auf den Aminosäureabbau* ausüben. Indessen scheint die Wirkung mit der Verschiedenheit der Substrate und der Gifte in mehr individueller Weise zu variieren.

Die Versuchsergebnisse, die hier gewonnen wurden, tragen also dazu bei, den Resultaten der Untersuchungen an Nieren- und Leber-Gewebe grössere Allgemeingültigkeit zu geben, ebenso wie sie vielleicht auch zum Verständnis der bekannten antibakteriellen Wirkung der Benzoesäure und ihr naheverwandter Stoffe beitragen können.

ZUSAMMENFASSUNG.

Zweck der vorliegenden Untersuchung war, den Einfluss der Benzoesäure, der Mandelsäure und nahverwandter Stoffe auf die Ammoniakbildung vor allem im Nieren- und Leber-Gewebe zu studieren.

Die Versuche, die mit Hilfe der Warburgschen Technik ausgeführt wurden, wurden hauptsächlich an Rattenorganen angestellt.

Anlass zur Untersuchung hatten gewisse klinische und experimentelle Beobachtungen gegeben, die dafür sprachen, dass die betreffenden Stoffe die Ammoniakbildung in den genannten Geweben hemmen.

Über diese Untersuchungen sowie über einige andere, die zeigten, dass die betreffenden Stoffe mehrere Prozesse im intermediären Stoffwechsel hemmen, wurde hier Bericht erstattet. Ausserdem wurden die wichtigsten ammoniakbildenden Prozesse in Niere und Leber diskutiert.

Der Hauptsache nach haben die Untersuchungen umfasst:

1. Einen Vergleich zwischen dem Einfluss der Benzoesäure und dem der Mandelsäure auf die spontane Ammoniakbildung im Nieren- und Leber-Gewebe.

Hierbei ging hervor, dass beide Stoffe eine ungefähr gleichgrosse, erhebliche Hemmung der spontanen Ammoniakbildung im Nierengewebe bewirken, während im Lebergewebe keine sichere Hemmung nachzuweisen war, was wahrscheinlich darauf beruht, dass die Leber die Fähigkeit hat diese Stoffe zu entgiften.

2. Einen Vergleich mehrerer Stoffe, die in verschiedener Weise mit Benzoesäure und Mandelsäure verwandt sind, um hierdurch die aktive Gruppe zu erkennen.

Hierbei ergab sich, dass es wahrscheinlich die Kombination von Phenyl- und Carboxylgruppe ist, die die Wirkung der Stoffe bedingt. Die beiden Gruppen scheinen nämlich in der Regel, jede für sich in Verbindung mit anderen Gruppen, eine Hemmung hervorzurufen, die schwächer ist als die, die sie bei gemeinsamen Auftreten in einer phenylsubstituierten Fettsäure bewirken.

3. Eine Untersuchung des Einflusses der Benzoesäure auf verschiedene ammoniakbildende Prozesse je für sich.

Hierbei ergab sich, dass die Benzoesäure, die als Repräsentant für die übrigen Stoffe verwendet wurde, gewisse Prozesse wahrscheinlich ganz hemmen kann, während andere nicht beeinflusst werden. So wird die Desaminierung von l- und d-Aminosäuren im Nierengewebe gehemmt, während die Desaminierung von Muskeladenylsäure und die Desamidierung von Asparagin nicht beeinflusst werden dürfte. Durch gewisse Abänderungen der Versuchsbedingungen ergab sich als wahrscheinlich, dass im Lebergewebe ähnliche Verhältnisse vorliegen, mit dem Unterschiede, dass die Leber, wie oben erwähnt, die in Rede stehenden Stoffe in einem gewissen Ausmass entgiften kann.

4. Eine Untersuchung des Einflusses der Benzoesäure auf die spontane Ammoniakbildung in Geweben von anderen Organen (Milz und Muskulatur).

Hierbei ergab sich, dass die spontane Ammoniakbildung durch Benzoesäure, die als Repräsentant für die übrigen Stoffe diente, nicht beeinflusst wird, was dann wahrscheinlich darauf beruht, dass die Aminosäuredesaminierung hier keine wesentliche Rolle spielt.

5. Eine Untersuchnung des Einflusses der Benzoesäure, Mandelsäure und Salicylsäure auf die Aminosäuredesaminierung bei Bacterium coli.

Hierbei ergab sich, dass sie gehemmt wird, wie es auch bei der Aminosäuredesaminierung im Nieren- und Leber-Gewebe der Fall ist.

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FROM THE NEUROPHYSIOLOGICAL LABORATORY AND THE NEUROLOGICAL CLINIC, KAROLINSKA INSTITUTET, STOCKHOLM.

ACCOMMODATION IN HUMAN NERVES

AND ITS SIGNIFICANCE FOR THE SYMPTOMS IN CIRCULATORY DISTURBANCES AND TETANY

By

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THESIS,
with the sanction of the Royal Caroline Institute, submitted to public defense at the Great Lecture Theatre of the Institute on May 9th, 1944, at 9.00 o'clock.

Stockholm 1944

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PREFACE.

The work presented in this book was taken up as a problem of collaboration between the Neurophysiological Laboratory and the Neurological Clinic. Accommodation has been studied for some time at the Neurophysiological laboratory with electrophysiological methods, and to the author was allotted the task of taking up the same theme with normal human material and with a view to the use of accommodation as an aid in the analysis of certain disturbances of nerve excitability.

It is a pleasure to record my gratitude to the Director of the Neurophysiological Laboratory, Professor RAGNAR GRANIT, for constant advice and criticism during the experimental work as well as to the Head of the Neurological Clinic, Professor NILS ANTONI, for placing the facilities of his clinic at my disposal and for instructive discussions of the results.

The Lecturer in Neurophysiology, Dr C. R. SKOGLUND, has freely assisted me with his experience in this field and Mr K. T. HELME, physicist to the Neurophysiological Laboratory, has several times checked the apparatus used in this work. To both of them I am greatly indebted for their valuable advice and help.

My thanks are due to Mr GRENVILLE GROVE for the translation of this treatise.

Stockholm, March 1944.

Eric Kugelberg.

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Introduction.

For some time, measurements of the strength-duration curve of excitation have been used in clinical work, generally in the abridged form of determinations of the chronaxie. But it would seem as if the concept of "excitability", as used by the clinician in examining human material, is in many respects more closely related to another parameter of excitability, namely, accommodation. This concept will be defined in the next section. There are relatively few studies of accommodation in man, none of them systematic enough to form a basis for a judgment of what could be achieved by measuring this parameter in an analysis of pathological states characterized by increased or diminished nerve excitability. They have been reviewed below (pp. 17) in SECTION I.

One of the main themes of the present work is to provide a set of data which might serve to show the variations and order of magnitude of "accommodation" in normal human subjects. This forms the substance of SECTIONS II and III, SECTION II in particular dealing with technique and procedure, SECTION III with the analysis of normal subjects.

These data having been procured, the work was extended into an attempt to use measurements of accommodation for analytical purposes. The work of LEWIS and his collaborators, supplemented by REID, ZOTTERMAN and others (see p. 52), has led to a great deal of information about selective losses of sensitivity and increased irritability ensuing upon circulatory disturbances caused by compression of the blood-vessels of the arm by pneumatic cuffs. Here therefore was an interesting possibility of finding out to what extent these changes could be better understood in terms of measurements of nerve accommodation. If changes in accommodation played a rôle in determining the phenomena observed under such circumstances, a convenient method for experimental modification of this factor would be available to provide a basis of comparison with changes in pathologically modified nerve. This surmise

proved correct. Rather remarkable changes in accommodation could be induced by circulatory disturbances produced in this manner, and it was found possible to correlate a number of symptoms of irritability, such as various paresthesias or muscle "fasciculation", as well as selective losses of excitability, with the accommodative changes and thus to comprise them under a common heading. The stretch of nerve in the proximal region of the arm was found in such experiments to possess interesting properties with regard to accommodation, just as previously this same region had been found to be of crucial significance for the phenomena described by LEWIS and his collaborators. These results are reported in SECTION IV.

Finally, in SECTION V, it was found possible by measurements of accommodation to throw new light upon certain pathological reactions such as paresthesia, spasms and fasciculations in tetany and the sign of Trousseau.

I. Historical section.

1. Accommodation: Definition of terms, methods of determination, investigations on animals.

Long before physiologists had succeeded in producing currents of sufficiently short duration to enable them to compute the time factor in excitation (chronaxie), it was known that a current which rises suddenly to its full value is more effective and excites at a lower threshold than if it rises slowly. LUCAS (1907 a) showed on toad and frog nerves that the current must have "a minimal gradient" in order to excite, and below which it has no effect, however strong it may at last have become. In the German literature gradually rising currents are designed as *"einschleichende"* (creeping), indicating that they approach the tissue so slowly that apparently they are not noticed at all. It thus looks as if the tissue, if allowed sufficient time, sets up a resistance against, and thus accommodates itself to, the stimulating current. Under the action of the current some process is apparently set up, which counteracts the stimulus and thus increases the threshold of excitation. This process, the nature of which still remains merely hypothetical, is termed accommodation (NERNST, 1908).

The simplest way of quantitatively measuring the accommodation is to stimulate with linearly or exponentially increasing currents and to determine the threshold strengths at different rates of current rise. If the accommodation is then rapid or good, the threshold — on stimulation by a current of a given rate of rise — increases more than if the accommodation is bad. A slowly rising stimulus must then be increased to a relatively greater strength in order to compensate the accommodation. In case the capacity for resisting the stimulating current is entirely lacking, the threshold will obviously lie at the same level, no matter how slowly the current rises. With a view to a comparison of the results from different objects of investigation, it is usual to refer the threshold values to a basic value by expressing them in multiples

of rheobasic strength. In this way an absolute measure of the accommodation can be obtained. This principle will be exemplified below by a method theoretically indicated by HILL (1936) and experimentally tested by his collaborator SOLANDT (1936 a).

It seems unnecessary to recapitulate all the experimental methods employed by investigators from v. KRIES (1884) onwards for quantitative determination of the speed of accommodation. Nor is it needful to discuss the various theoretical derivations of the time constant of accommodation, such as those made by RASHEVSKY (1933), MONNIER (1934) and HILL (1936). The constants, which have been computed theoretically, stand in a simple mathematical relation to one another. Similarly, as shown by HILL (1936), the results of the different experimental methods can be expressed in one and the same constant. For a more detailed description, the reader is referred to HILL's work (1936), to summaries by SCHAEFER (1940) and, especially as regards excitation with linearly increasing currents, to the paper by SKOGLUND (1942). Here it will suffice to describe the methods which have been adopted in investigations on man.

Seeing that the method adopted in this study is modelled on that of HILL-SOLANDT, it seems desirable at this point to give a brief description thereof. It is explained in greater detail on p. 23. SOLANDT employed currents with an exponential rise, which could easily be produced by discharging a condenser over a resistance (cf. use of this method by LAPICQUE 1908). Since the rising time of the current is proportional to the product of the condenser's capacity and the resistance, the desired rising times could be obtained by varying one of these factors. The rheobase is first determined with a muscle contraction as index of an excitatory effect, then the threshold strengths of currents with longer and longer rising times. The strength of the threshold currents is then divided by the rheobase and plotted against the time-constants of the respective currents (see Fig. 1.). A typical set of readings will be found on p. 25 and a curve traced according to these values on p. 30.

The result is a so-called accommodation curve, which for short rising times is linear. The upper steep curve in Fig. 1 shows a good accommodation, as the relative threshold increases rapidly in proportion to the rate of current rise, whilst a poorer accommodation is shown by the lower, more level curve. In this paper the slope itself has been taken as a measure of the accommodation,

whereas HILL-SOLANDT use the reciprocal, which is identical with the time-constant λ of accommodation theoretically derived by HILL. Thus, expressed in λ, the accommodation is, instead, good if λ is small, and bad if λ is large.

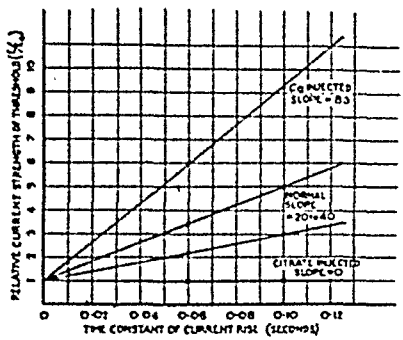


Fig. 1. From Solandt (1936 b). See text.

A technique based on the same principle has been employed by SCHRIEVER (1932) in order to obtain a gauge of the accommodation; which he terms *"Einschleichzeit"* or, in analogy with

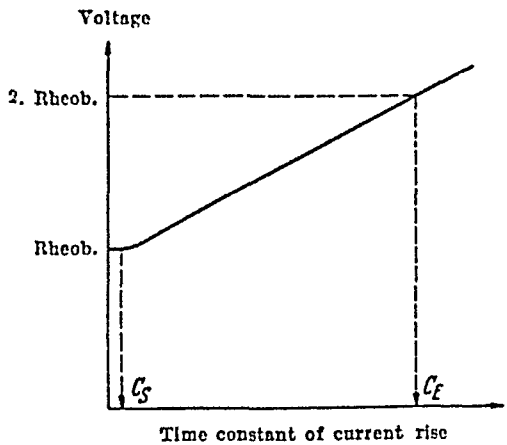


Fig. 2. Modified from Schriever and Cebulla (1938). Threshold voltage for muscle contraction as ordinates against time constant of exponentially rising stimulating current as abscissae. CS corresponds to *seuil de climalyse*. CE time constant for calculation of *Einschleichzeit*.

the chronaxie, *"Chronherpie"* (see Fig. 2). He first determines the rheobase and then the time-constant for an exponentially rising current which just suffices to excite at twice the rheobase. The *"Halbzeit"* for this current, i. e. 0.693 times its time-constant, is *"Einschleichzeit"*. As shown by HILL (1936), λ is 2.89 *"Ein-*

schleichzeit". Another gauge has been proposed by LAPICQUE (1937). As we know from the studies of FICK (1863), v. KRIES (1884) and GILDEMEISTER (1904), etc., there is a certain margin within which the rising time of a current of rheobasic strength can be prolonged without losing its effect. LAPICQUE determines the time-constant for the exponentially rising current which just prevents excitation by a current of rheobasic strength. This time-constant he terms *"seuil de climalyse"* (see Fig. 2), and has proposed its adoptation as a gauge of the accommodation. In practice, however, it is difficult to determine it precisely, seeing that, as shown by Fig. 2, the threshold curve quite gradually bends away from the abscissa, whence a considerable error in the rate of current rise will result unless the threshold strength is determined exactly, which, however, is practically impossible.

Also linearly increasing currents have been used in investigations on man by FABRE (1928), who determined his *"constante linéaire"*, which is the rheobase divided by the minimal current gradient. As shown by HILL (1936), this time factor is identical with λ.

There are, however, certain difficulties and complications in the computation of the accommodation. In order to obtain an exact measure of the slope of the accommodation curve, it must be linear, which in fact is usually the case as regards excised frog nerve, the most frequently used material for investigations of this nature. However, L. and M. LAPICQUE (1937), especially under the action of cold or after decalcification, but also spontaneously — similarly as P. and M. BENOIT (1937) after decalcification — have observed curves of another shape. These curves were at first linear and fairly steep, but turned round and flattened when the stimulating currents rose more slowly. Finally, they took a horizontal course when the muscular response to the stimulus was a tetanus, indicating that the nerve had responded with repetitive impulses. SCHRIEVER and CEBULLA (1938), in experiments on frog nerve which, by various measures, had acquired a tendency to "repetitiousness", found it to be a general rule that the curves tend to become horizontal when the nerve discharges repetively.

In a series of investigations on mammalian nerves *in situ*, BERNHARD, GRANIT and SKOGLUND (1942), SKOGLUND (1942) and GRANIT and SKOGLUND (1943) have studied the nerve response to slowly rising rectilinear currents. BERNHARD, GRANIT and SKOGLUND (1942) have regularly found that the accommodation curves

gradually become horizontal where the relative current strength is sufficiently great, in which case the nerve begins to respond to the stimulus by repetitive impulses, which can be observed by concurrent recording of the muscular or nerve action potentials. This indicates that the nerve can adapt itself to the stimulus only within a certain range of current strength, beyond which this capacity is lost, or, in other words, a "breakdown of accommodation" occurs. Mammalian nerves thus lack a "minimal current gradient" (the "*pente limite*" of the French authors): the currents excite, however slowly they may rise, provided that their threshold values exceed the limit at which the "breakdown" takes place.

In view of this fact, only the initial part of the curve, where it is linear, should be used in determining the accommodation. The linearity should also be controlled by determining additional points, as deviations from the linear course at the very beginning of the curve have been observed by SKOGLUND (1942) in some measurements of accommodation in mammalian nerves, as well as e.g. by KAHLSON and v. WERZ (1936), SOLANDT (1936 a) and L. and M. LAPICQUE (1937) in frog nerves.

Another complication, as shown by SKOGLUND (1942), is the uncertainty of the index hitherto employed, namely a muscle contraction. That author has elaborated a new technique, based on using as an index the first action potential observed on the stimulation of the nerve. By this procedure several sources of error are avoided, besides which it permits of the examination also of sensory nerves. The form of the stimulating current is recorded photographically by the one beam of a double cathode ray oscillograph, whilst the nerve impulses are recorded by an amplifier connected to the other beam. With this method the accommodation in nerve fibres with the lowest threshold can be ascertained. It was further found that the determination of the accommodation partly depended on what index was employed. Thus the slope of the accommodation curve will be steeper if one selects as an index a large muscular or nerve spike — which probably consists of synchronized impulses from several fibres — than if a small spike with little or no synchronization is selected for the purpose. The actual accommodation in the nerve fibre can thus be ascertained only if, as suggested by SKOGLUND (1942), the first nerve action potential is used as an index in relatively isolated fibres, or still better if, as done by GRANIT and SKOGLUND (1943), the

same method is employed directly on single fibres isolated by micro-electrodes. If a muscle contraction is used as an index, the accommodation will always be determined to some extent by a component of unknown magnitude, which depends on the synchronization in the nerve examined. Moreover it seems that the synchronization can most easily be established on stimulation with currents of short rising times. The index is therefore liable to change and to consist of less well synchronized impulses according as the thresholds of longer and longer rising times are determined. This is an additional reason for checking the linearity of the curve, in order to make sure that the index has been kept constant. Thus the chief difficulty in using a threshold muscle contraction as an index is the variability of that index.

With the use of the above-mentioned technique for the stimulation of separately prepared cat nerve *in situ* with linearly increasing currents, SKOGLUND (1942) found that the accommodation in the motor nerve showed relatively little variation from individual to individual. He ascertained also, as ERLANGER and BLAIR (1938) had previously found in experiments on frog nerve with quite a different technique, that sensory fibres, in this case presumably muscle proprioceptors, show a poorer accommodation than motor fibres. The former showed an accommodation, as expressed in λ (inverse value of slope of accommodation curve), ranging between 90 and 200 msec., the latter between 30 and 60 msec. In the sensory fibres the breakdown of accommodation occurred at 1.5—2 times the rheobasic strength, in the motor fibres at 3—4 times that strength. As will be explained below, this signifies that the threshold of the sensory fibres for prolonged "repetitiousness" lies lower than for the motor fibres, as has also been found by ERLANGER and BLAIR (1938) in frog nerves.

The capacity for accommodation can be artificially modified within wide limits. LUCAS (1908) thus showed that the accommodation of frog muscle was retarded if it was bathed in calcium-free Ringer's solution, but recovered its rapidity on the addition of calcium chloride. This effect of the lack of calcium ions on the accommodation in muscle was confirmed by KAHN (1911). SOLANDT (1936 a), in similar experiments on nerve, found that the accommodation was retarded if the nerve was deprived of calcium ions (e. g. by bathing it in calcium-free Ringer's solution, by the addition of oxalate or citrate, or by shifting pH to the alkaline side), but was immediately improved by the addition of a

calcium salt or by increased acidity (see Fig. 1). Thus, simply by changing the calcium ionization in the environment of the frog nerve, he could vary λ from 6 msec. to infinity. Furthermore, as shown by LIESSE (1938, a and b), the rapidity of accommodation in frog nerve is increased by ischemia. According to KAHLSON and v. WERZ (1936) and SCHRIEVER and EHRHARDT (1939), a similar effect is produced by certain narcotics. Cooling of the frog nerve or maltreatment by drying or mechanical injury have the opposite effect (SCHRIEVER and CEBULLA, 1938). There are also some other factors which have been stated to affect the accommodation; but those above enumerated seem to be the principal ones that call for mention from the special viewpoints of the present study.

The importance of the concept "accommodation" lies also in the fact that it provides a simple, measurable expression for a fundamental physiological property in the nerve, namely its tendency to respond with repetitive impulses to a stimulus. This tendency is quite intelligible: for, if the nerve cannot accommodate itself to a stimulus above threshold strength, its effect continues, whence the nerve, so long as the stimulus is applied, should respond with a prolonged series of such impulses. But, if the accommodation is rapid or good, the stimulus is quickly counteracted, and we should obtain merely a single impulse or a short series of impulses.

That accommodation is an important factor controling the tendency of the nerve to "repetitiousness" has been shown by HILL's associate KATZ (1936, a and b). Thus nerves of cooled frogs, nerves which have been deprived of their ionized calcium as well as nerves of crabs and lobsters, all respond with repetitive impulses to constant current at, or slightly more than, rheobasic strength. Such nerves show little or no accommodation. If calcium is added, the current must be made stronger in proportion to the improvement in accommodation, in order to obtain a repetitive response to the stimulus. SCHRIEVER and CEBULLA (1938) have confirmed this in experiments on frog nerve. Another example has already been mentioned. As soon as a breakdown of accommodation was observed on determining the course of the accommodation curve, the nerve began to respond iteratively to stimuli beyond the threshold of the breakdown.

Another phenomenon which is closely associated with bad or non-existing accommodation and the consequent greatly increased

2. Studies of accommodation in man.

As compared with the very large number of investigations bearing on the chronaxie in man, accommodation, in the meaning assigned to that term here, has been a rather neglected subject. The first quantitative determinations under normal conditions were made by FABRE (1928), who computed his *"constante linéaire"* on a motor point in different arm and leg muscles of some individuals. His values ranged between 25 and 60 msec. In a later work (1935), however, he contended that a *"constante linéaire"* could be computed for currents stimulating nerves but not muscles, as the latter apparently lacked a well-marked *"pente limite"*. The current in fact finally stimulated the muscle, quite irrespective of its rate of rise, provided that it had acquired sufficient intensity. It should be noted, however, that FABRE's investigations were made chiefly on excised frog nerve, the curves of which, as previously pointed out, generally show a *"pente limite"* or "minimal current gradient" — the basis of FABRE's method of determination in the form indicated by him — whereas a minimal current gradient is lacking in the curves for mammalian nerves. (See the preceding chapter). The only complete accommodation curves which have hitherto been recorded in investigations on man, namely those of LIBERSON (1934), likewise lack a minimal gradient.

Thus presumably it is not possible to determine a *"constante linéaire"*, as defined by FABRE, for the nerves and muscles of man. The values given by FABRE for the accommodation, however, are in good correspondence with later investigations, presumably because the stimulating current was not sufficiently strong to break down the accommodation. Hence, as in other methods of determination, the accommodation was actually computed from the linear initial part of the accommodation curve.

SCHRIEVER (1932) computed his *"Einschleichzeit"* at the motor point of the *musculus biceps* of 6 individuals. The values ranged between 13.0 and 32.1 msec. The largest normal material, however, has been collected by SOLANDT (1936 a), who computed the time-constant of accommodation λ on the *nervus ulnaris* with the electrode at the elbow, and with a minimal palpable twitch in the tendon of the *musculus flexor carpi ulnaris* as an index of the excitatory effect. His material comprised 13 men and 3 women.

on two healthy subjects, in fact succeeded in reducing λ from 55—60 to 37—45 msec. by experimentally induced acidosis, resulting from the administration *per os* of 10 gm $CaCl_2$ and 10 gm NH_4Cl. Finally, an administration of 60 gm sodium carbonate caused λ to rise from 70 to 140 msec.

In muscle degeneration due to peripheral nerve lesions and in myotonia, qualitative changes in the reaction to stimulation with slowly rising currents have long been known. Thus REISS (1911), LAPICQUE (1915, 1940) and others, have established that a muscle which shows a reaction of degeneration responds to the stimulus in the shape of a slowly rising current at a threshold lying near that of the instantly rising current, which signifies that the accommodation is slow. This observation, however, has been little utilized in diagnostics. LAPICQUE (1915, 1940) has also pointed out the desirability of using slowly rising currents in electrotherapy, which facilitate selective stimulation of the injured muscle, because the normal muscle, owing to its more rapid accommodation, will not respond to the stimulus. In this way disturbing twitches of healthy muscles will be obviated, besides which the slowly rising current is less painful.

The myotonic muscle has no capacity for accommodation, as has been shown by PÄSSLER (1906) and afterwards by KRAMER and SELLING (1912), etc. This fact has acquired greater interest since BROWN and HARVEY (1939), in experiments on myotonous goats, and BUCHTHAL and CLEMMESEN (1941), in man, have found that the myotonic contraction and the myotonic reaction to artificial excitation is accompanied by a series of action potentials, as in tetanic muscle contraction: a repetitive response to excitation is in fact rendered possible by the slow accommodation. — The study of neurogenous muscle degeneration or of the myotonic muscle is, however, beyond the scope of this paper.

It should be mentioned that some of the results reported here have been briefly summarized in the proceedings of the Swedish Neurological Society, 1943.

II. Technique of measurement of accommodation.

1. Stimulating apparatus.

As stimulating currents of different rates of rise, exponentially increasing currents have been selected for two reasons. Firstly, they are, technically speaking, much easier to produce than linearly increasing currents; and secondly, nearly all the material for comparison has been obtained with currents of the first-mentioned kind.

The stimulator[1] used for this purpose has been constructed by Mr K. T. HELME, the physicist of the Neurophysiological laboratory. In its design, due regard has been paid to the desirability of reducing, as far as possible, the effect of the resistance of the skin, and of changes in that resistance, on the form and strength of the stimulating current. A description of the apparatus has been published by GRANIT and SKOGLUND (1942), in the Swedish language. It is shown in outline in Fig. 3.

The principle on which the apparatus operates is briefly as follows. In rest the anode current from the pentode valve (P) is completely blocked by a just sufficiently strong negative potential on the grid. If the contact (B) is closed and no condenser (C) is connected to the circuit, the grid of the pentode valve is immediately charged and a constant anode current with the rising time 0 is obtained at the stimulating electrodes (E). If the contact (B) is closed while a condenser (C) and a resistance (R) are connected to the circuit, the grid will not be charged immediately, but after a time-lag, as the potential in the condenser rises exponentially with a time-constant CR. (If C is the capacity of the condenser in microfarads and R the resistance in thousands of ohms, the constant CR is obtained in msec.) Thus, by connecting a condenser of suitable capacity in the grid

[1] The stimulator was built by the firm Svenska Radioaktiebolaget, Stockholm.

circuit, the required rising time can be obtained. This principle has previously been applied, for example, by KAHLSON and v. WERZ (1936). In order to obviate deformation of the stimulating current owing to the non-linear properties of the valve, a resistance (D) of sufficient value has been connected in the lead to the cathode of the valve.

In order to avoid stimulation on the breaking of the current, the condenser (C) is discharged over the resistance R + R′, which

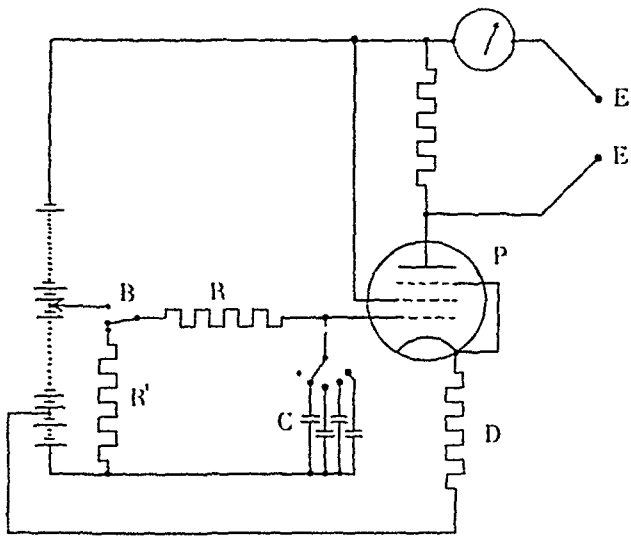


Fig. 3. Diagram of stimulator. Description in text.

causes the current to fall exponentially by a time-constant which is larger than when it rose. The apparatus is designed for operation by alternating current and for connection to the mains.

Owing to the high internal resistance of the valve employed (EF 14. Telefunken), viz. about 150 000 ohms, as well as the resistance of the shunt of 100 000 ohms (which is high relatively to the resistance of the skin and the tissues), the anode current is forced as to more than nine-tenths through the stimulating electrodes to the tissues. Owing to this arrangement, the form or strength of the stimulating current will be less affected by changes in resistance or by the polarization and capacity in the skin than would otherwise be the case. In the next chapter, these factors have been experimentally analyzed.

Only monopolar stimulation has been employed, with the cathode as the "active", and the anode as the "indifferent", electrode.

The cathode consisted of a thin plate of silver at the bottom of a thin, round, cup-shaped ebonite cap, filled with asbestos. Its diameter was 1.5 cm for stimulation of the nerve and 2 cm for stimulation of the muscle. As an indifferent electrode, a thin silver plate of 9×10 cm², covered with chamois leather, was employed. It was fixed with a gauze bandage on the forearm or leg. Both electrodes were moistened before use with a 4 % sodium chloride solution.

2. Control of stimulating apparatus.

The form of the exponentially rising currents is expressed by the equation:

$$I = I\infty\,(1 - e^{-t/k}),$$

where I is the intensity of the current at the moment t, $I\infty$ the final value of I at $t = \infty$, k the time-constant of rise of I, and e the base of the natural logarithm (2.718 approximately). Putting $t = k$, we obtain $I = \frac{63.2}{100} I\infty$, whence the time-constant of rise is the time taken by the current to acquire 63.2 per cent. of its full value; k is = RC.

Currents of different time-constants and strength were recorded photographically with a cathode ray oscillograph and the beam was made intermittent with a Philips' sinus current generator, for the purpose of time records. (Fig. 4.)

The frequency of the sinus generator was calibrated against the alternating current of 50 periods in the mains. Photographic records were taken with a non-inductive resistance of 1 000—20 000 ohms and with the experimental subject connected, as in measurement of accommodation in the circuit, in series with the oscillograph. No differences in the form or time-constants of the currents could be observed, whence a resistance of 1 000—20 000 ohms, or the resistance of the skin, has no effect on the form or rate of rise of the current. The records moreover show that, when the current has acquired its full strength, it remains constant sufficiently long for its strength to be read on the meter. The strength of the current read is thus independent of any variations in the resistance of the body which may have been caused by the current. This observation has been controlled as regards the current strengths usually employed, viz. 0.25—5 mA.

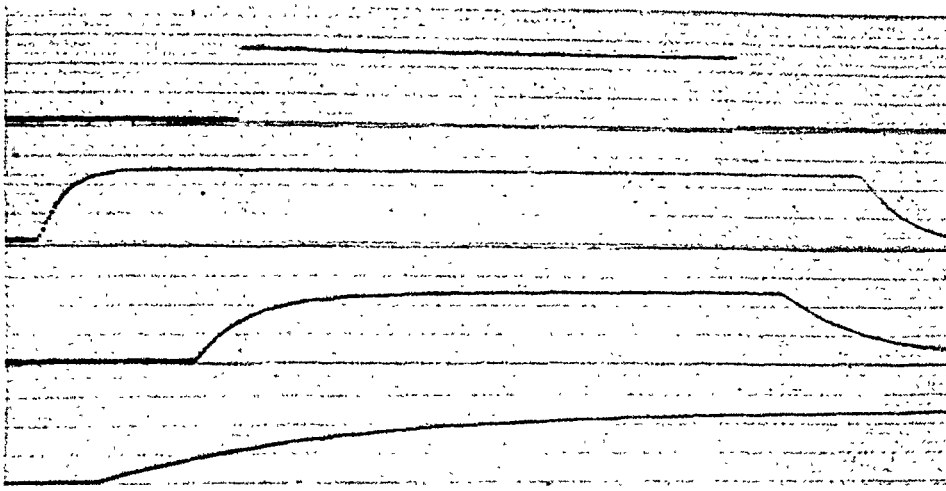


Fig. 4. Oscillograms of rheobase and exponentially rising stimuli. Subject in series with stimulating electrodes. Uppermost curve rheobasic stimulation, the following curves taken with the time constants 10.21 and 98 msec.

The time-constants computed from the records give the same result within 5 per cent. as if they had been calculated as RC. Finally, the theoretical exponential curve of a time-constant, selected among those obtained from the photographic records, was constructed. The photographically recorded curve coincides with the ideal curve, except for an insignificant distortion due to the valve. Moreover the curve which shows the relation between grid potential and stimulating current, is practically linear, which likewise indicates that this distortion is negligeable.

3. Procedure.

Most of the measurements of accommodation recorded in this paper were made on nerves and muscles in the arm. The experimental subject sat at a table opposite the experimenter and usually made the records himself with his right arm: hence it was chiefly the left arm that was examined. No other assistance is required in these experiments.

A nerve or motor point is first selected, whereupon the "active" cathode is fixed on it with one or two strips of plaster. It had in fact been found that if, as in measurements of the chronaxie, one endeavours to hold the electrode manually, it will be difficult and laborious to obtain good accommodation curves, besides

which further assistance is then usually required. The fixation of the electrode in this manner does not indeed afford any reliable guarantee that it will not change its position during the course of the measurement. Rheobase controls made during the measurement show, however, that dislocations of the electrode are unusual, provided that the part of the body in which it has been fixed is kept perfectly still.

In certain circumstances, however, the electrode cannot be fixed with strips of plaster. In the stimulation, especially in corpulent individuals, of deep-lying nerves such as the tibial nerve, or of the brachial plexus and radial nerve in muscular persons, it will be found necessary to press in the electrode somewhat by hand, in order to avoid the use of strong and painful stimulating currents.

In such experiments due regard should also be paid to the fact that the accommodation is rapidly changed by disturbances in the circulation: the pressure should therefore be weak and be applied each time for some tens of seconds only.

Importance has been attached to uniformity in the measurements. Thus all thresholds were determined by stimulating with currents which were being gradually increased in strength. Sufficiently long pauses were made between each stimulation: with weak currents, as is generally known, the thresholds are usually determined too low, if the stimuli follow closely on one another. On the other hand, when strong currents of several times the rheobase are used, especially under pathological conditions, the stimulus is usually followed by a decrease of excitability which lasts several seconds: the thresholds will therefore, instead, be too high if a sufficient length of time is not allowed to elapse between each stimulus.

When the "active" electrode can be fixed with strips of plaster, as is generally the case, a desired number of points on the accommodation curve can easily be determined. A typical example is shown in table II. The curve traced in accordance with these data will be found in Figs. 5 and 6.

The rheobase is first determined, and then the thresholds of currents of increasing time-constants. The value of the thresholds is read in mA. At suitable intervals, as indicated by the typical example, the experimenter should make sure that the rheobase has not changed. Should the difference between two succeeding determinations of the rheobase amount to more than 10 per cent.,

Table II.

Example of data obtained by exciting the ulnar nerve at the elbow. Index of nerve excitation: minimal twitch observed in musc. inteross. dors. 1.

RC. (m. sec.)	Threshold (m. A.)	Threshold / Rheobase
0	1.5	1.00
10	1.6	1.07
21	1.9	1.27
30	2.1	1.40
0	1.5	
42	2.4	1.60
48.5	2.6	1.73
0	1.5	
73	3.1	2.07
98	3.6	2.40
200	4.6	3.07
300	5.0	3.33
0	1.5	

Initial accommodation slope = 17.2

the intermediate determinations of threshold should be repeated. In certain pathological conditions, however, such as neurogenous muscle degeneration and tetany, where the thresholds spontaneously fluctuate far more than normally, such precision cannot be maintained unless the rheobase controls are made at more frequent intervals than in the typical example.

The absolute value of the thresholds indicates merely the current strength which passes the stimulating electrodes. What actually passes the stimulated tissue is, of course, unknown and depends on a number of variable factors, such as the distance from the electrodes, etc. As the distribution of current during a series of observations should be assumed to remain unchanged, the relation between the current which actually stimulates and that which flows through the electrodes is constant. In order to obtain a quantitative value, the thresholds of the exponential currents are divided by the rheobase. In tracing the accommodation curve, the relative strength of current is plotted on 1 mm square-ruled paper as an ordinate, with the time-constant of the current as the abscissa. The slope of the linear part of the curve is taken as a gauge of the accommodation. In practice it has been determined by stimulation with currents of the time-constants 10, 21, 30, 42 and 48.5 msec., except in cases where the electrode

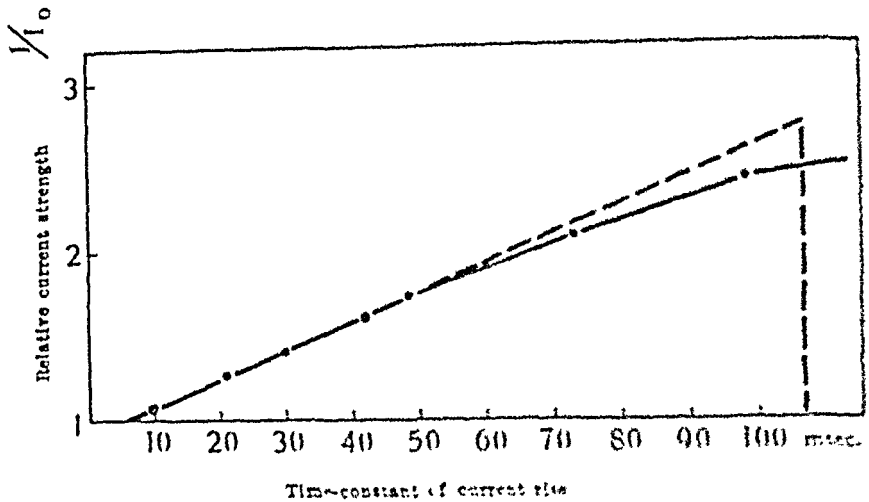


Fig. 5. Diagram illustrating calculation of "accommodation slope". The rectilinear portion of the curve has been prolonged to correspond to 100 msec., as calculated from ordinate 1, which represents rheobasic strength. The ordinate drawn in broken lines shows the number of rheobasic units per 100 msec. and corresponds to 17.2 rheobasic units per sec.

could not be fixed. The slope was then computed by repeated determinations of the threshold for the stimulating current of 48.5 msec., with rheobase controls before and after each determination.

The slope was computed by prolonging the rectilinear portion of the curve and graphically reading by how many rheobases this linear part had risen in 100 or 1 000 msec. (see Fig. 5). In this paper the slope has been defined as the number of rheobases per 1 sec. If it is desired, instead, to express the accommodation in λ and msec., one should take the inverted value of the slope, multiplied by 1 000.

4. Accuracy of the method.[1]

In order to compute the standard error of the method, double determinations were made, in the course of the work, on 21 healthy subjects (see table III). The first 11 determinations recorded in the table were made on the ulnar nerve, stimulated somewhat proximally of the elbow, with a minimal twitch in the tendon of *M. flexor carpi ulnaris*, palpable at the wrist, as an index. The

[1] I am indebted to Dr Leonard Goldberg for assistance in the statistical treatment of the data.

10 following determinations were made by stimulating a motor point of *M. flexor digitorum sublimis, dig.* 3—4, with a minimal observable movement of the finger as an index. The electrode was removed after the first determination, and a nerve or motor point was again selected after the lapse of a few minutes. No statistically significant difference was found between the first and second determination.

Table III.

Double determinations of initial accommodation slope.

Case	Determination I.		Determination II.	
	R	Slope	R.	Slope
1	1.1	19.5	0.8	17.8
2	Discarded owing to breaks in the curve.		—	—
3	1.3	20.6	1.2	21.5
4	1.0	19.0	1.2	17.8
5	1.2	18.4	1.2	17.0
6	1.3	25.4	1.7	25.8
7	1.5	26.5	1.5	23.8
8	1.5	27.5	1.5	29.0
9	1.4	23.0	1.5	24.7
10	0.9	16.4	1.2	17.0
11	1.1	18.5	1.0	18.3
12	0.9	19.7	0.8	18.6
13	2.2	23.4	1.8	24.8
14	1.2	23.5	1.3	22.7
15	1.5	22.0	1.4	24.6
16	2.0	22.0	1.8	22.5
17	1.5	25.4	1.8	27.4
18	0.5	14.5	0.6	13.5
19	2.2	28.8	2.5	26.3
20	1.9	22.2	1.2	22.2
21	1.7	26.5	2.0	28.2
				Mean 22.2

Standard error of a single determination ± 1.09 or ± 4.9 % of the mean.

The accuracy of the method was 5 per cent.

5. Effect of position of stimulating electrode relatively to nerve.

As most of the determinations recorded in this paper were made at a nerve point, it is technically of interest to ascertain whether the accommodation is independent of the position of the electrode relatively to the nerve, and thus independent of what

fraction of the rheobase is shunted. For this purpose the accommodation was computed in 2 individuals, alternately at a nerve point and a few centimetres to the side of it, in the ulnar nerve at the elbow. A minimal palpable twitch in the tendon of *M. flexor carpi ulnaris* was taken as an index. Five double determinations were made on each experimental subject. See table IV.

Table IV.

Initial accommodation slope obtained on excitation of a »nerve point» and at a distance of a few centimetres from it.

Nervus ulnaris excited at the elbow. Index: minimal palpable twitch in the tendon of *musc. flexor carpi ulnaris.*

Case	At the "nerve point"		At a distance from the "nerve point"	
	R.	Slope I	R.	Slope II
1	0.6	25.0	1.0	24.8
	0.6	22.6	1.8	21.5
	0.7	23.0	1.4	20.8
	0.7	22.4	4.3	21.8
	0.7	21.2	1.2	23.2
2	1.2	24.7	1.9	25.2
	1.1	20.8	3.6	24.6
	1.4	26.5	2.8	24.7
	1.3	25.8	1.9	26.3
	1.1	25.9	4.5	26.5

Mean difference of slopes (II—I) . . . . . + 0.15

Standard error of mean difference 0.57

Thus there is no statistically significant difference between the values obtained on stimulation at a nerve point or beyond it. However, one should always endeavour to stimulate at a nerve point in order to avoid unduly strong currents. Besides, it is then easier to keep the rheobase constant during the determination, as a minor dislocation of the electrode at a nerve point appears to result in a smaller change of threshold, than is the case if the electrode is placed outside that point.

III. Accommodation under normal conditions.

A. Motor Nerves.

1. The accommodation curve.

Typical accommodation curves from different nerves are shown in Fig. 6.

The curve leaves the abscissa at a point which is the "*seuil de climalyse*" of LAPICQUE. It then takes a linear course and rises rather steeply. The accommodation is thus good. In such cases the nerve adapts itself rapidly to the stimulus, whence the muscle response will be a short distinct twitch, the threshold of which can be easily observed.

For longer rising times (normally not less than 50 msec.), the curve gradually bends away and takes a more horizontal course. In the terminology of BERNHARD, GRANIT and SKOGLUND (1942), the accommodation is broken down. The curve, however, never becomes quite horizontal: thus some capacity for accommodation always remains, though the stimulus can no longer be counteracted so rapidly; pending such adaptation, the nerve responds with iterative impulses, whence the muscle response is a slow contraction or, more strictly, a brief tetanus. In such cases it is difficult to determine the threshold exactly.

Though it is not possible to show exactly on the curve at what relative strength of current the breakdown takes place, this is evidently a factor which normally varies within relatively narrow limits. Where the stimulating currents range between 2.5 and 5 times the rheobasic strength, the curve definitively takes a more horizontal course. With a few exceptions, there is a close connection between the slope of the accommodation curve and the breakdown: the slower the accommodation, the sooner does the breakdown occur. On stimulation of the facial nerve, however, steep slopes showing a breakdown at 2.5—3 times the rheobasic strength are sometimes observed. (For further particulars see p. 38.)

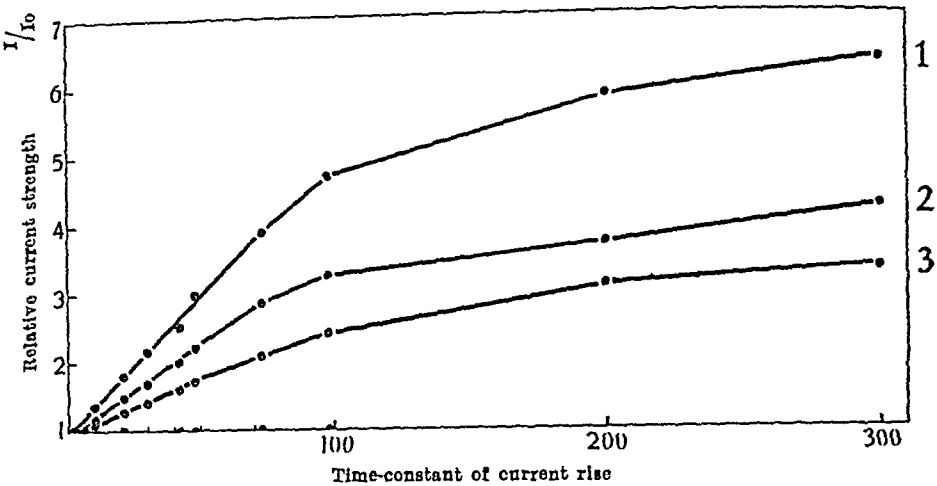


Fig 6. Typical accommodation curves from motor nerves. 1) *n. facialis-musc. orbicularis oris.* Initial slope 40.5. 2) *n. ulnaris*-tendon twitch of *musc. flex. carp. uln.* Slope 26.6. 3) another subject, *n. ulnaris-musc. inteross. dors.* 1. Slope 17.2.

It may be asked in what relation the threshold of the cathodal closing tetanus stands to the breakdown. From a practical, clinical point of view, the cathodal closing tetanus may be defined as the threshold of a suddenly rising current strong enough to elicit iterative impulses of sufficient duration to be observed as a tetanus. The violent initial contraction in the muscle, however, greatly impedes the observation of the tetanus, whence the determination of its threshold will be somewhat arbitrary. It is also clear that suddenly rising currents of a strength exceeding the threshold for the breakdown elicit a tetanus, as they can no longer be so rapidly counteracted by accommodation. It may thus be stated that the threshold of the cathodal closing tetanus, in general, cannot exceed the threshold for the breakdown. In all my experiments the values of the cathodal closing tetanus and the breakdown of accommodation have approximately corresponded. The cathodal closing tetanus therefore also lies between 2.5 and 5 times the rheobasic strength.

2. Limits of variations.

The accommodation slope was determined in tests on 100 healthy subjects, 50 men and 50 women, at the ulnar nerve, which was stimulated somewhat proximally to the elbow, with a palpable twitch in the tendon of *musc. flexor carpi ulnarias* index. The "slope"

Table V.

Normal values of the initial accommodation slope recorded for healthy male subjects. Determined on Nervus ulnaris excited at the elbow. Index: minimal palpable twitch in the tendon of musc. flexor carpi ulnaris.

Case:	Date:	Age:	Rheobase:	Initial slope:
1	22. 7. 1942	26	2.5	31.2
2	9. 10. »	25	1.5	30.5
3	10. 10. »	36	1.5	26.0
4	26. 9. »	25	1.1	24.8
5	15. 9. »	25	1.2	24.8
6	22. 7. »	25	1.6	24.5
7	8. 4. »	27	1.5	24.4
8	14. 4. »	25	1.4	24.4
9	10. 4. »	29	2.0	24.0
10	15. 9. »	25	1.1	24.0
11	9. 10. »	25	1.5	24.0
12	22. 7. »	31	2.3	23.5
13	9. 10. »	24	1.1	23.5
14	14. 4. »	32	1.3	23.3
15	26. 9. »	23	1.3	23.3
16	14. 4. »	26	0.75	23.1
17	16. 9. »	24	2.3	23.1
18	9. 10. »	24	1.4	22.6
19	14. 4. »	24	1.1	22.0
20	24. 7. »	26	1.3	22.0
21	26. 9. »	35	1.2	21.8
22	11. 4. »	27	0.9	21.6
23	16. 9. »	24	2.1	21.4
24	22. 9. »	26	0.7	21.0
25	18. 9. »	30	1.9	20.8
26	14. 4. »	30	1.9	20.4
27	11. 4. »	26	1.2	20.3
28	11. 4. »	24	0.5	20.2
29	13. 4. »	25	0.7	20.0
30	23. 7. »	26	1.7	20.0
31	15. 9. »	26	0.8	19.6
32	29. 4. »	25	1.5	19.6
33	18. 9. »	25	1.1	19.5
34	10. 4. »	23	1.1	19.3
35	9. 10. »	25	1.5	19.2
36	30. 7. »	25	1.0	18.9
37	22. 7. »	24	1.3	18.6
38	24. 7. »	25	0.7	18.5
39	9. 10. »	26	1.4	18.4
40	24. 9. »	29	1.7	18.3
41	9. 10. »	25	0.9	18.2
42	24. 9. »	26	1.1	18.1
43	26. 7. »	25	1.2	17.8
44	10. 4. »	24	0.7	17.4
45	23. 4. »	37	1.4	17.4
46	16. 9. »	23	1.3	17.3
47	14. 9. »	27	0.36	17.2
48	30. 5. »	23	0.9	17.0
49	18. 9. »	39	0.9	16.9
50	14. 4. »	26	1.4	16.6

Mean value 21.2
Standard deviation 3.72
Standard error of the mean.. 0.46

Table VI.

Normal values of the initial accommodation slope recorded for healthy female subjects. Determined on Nervus ulnaris excited at the elbow. Index: minimal palpable twitch in the tendon of musc. flexor carpi ulnaris.

Case:	Date:	Age:	Rheobase:	Initial Slope:
1	11. 9. 1942	23	1.1	26.4
2	7. 4. »	24	1.5	25.6
3	12. 9. »	21	2.6	25.0
4	31. 10. »	22	1.5	25.0
5	10. 4. »	23	2.3	24.6
6	24. 7. »	25	1.9	24.5
7	31. 10. »	20	2.0	24.5
8	19. 11. »	30	1.4	24.5
9	12. 9. »	20	2.3	24.3
10	9. 4. »	27	1.5	24.2
11	11. 9. »	24	2.1	24.1
12	7. 4. »	25	1.5	23.8
13	11. 9. »	21	1.1	23.8
14	11. 9. »	25	1.7	23.6
15	11. 9. »	31	2.0	23.3
16	24. 9. »	28	1.1	23.0
17	12. 9. »	23	1.0	22.8
18	28. 10. »	20	1.1	22.5
19	26. 10. »	23	1.1	22.5
20	11. 9. »	27	1.4	22.4
21	11. 9. »	23	2.6	22.0
22	19. 11. »	25	1.4	22.0
23	28. 10. »	25	1.5	21.8
24	11. 9. »	24	2.3	21.5
25	4. 10. »	24	1.5	21.4
26	4. 10. »	31	1.7	21.3
27	12. 9. »	20	1.8	21.2
28	14. 4. »	31	1.3	21.1
29	12. 9. »	45	1.5	21.0
30	14. 4. »	19	1.5	20.8
31	4. 11. »	23	1.4	20.8
32	12. 9. »	27	1.3	20.7
33	12. 9. »	20	1.2	20.6
34	21. 10. »	22	1.1	20.5
35	19. 11. »	29	1.0	20.5
36	19. 11. »	21	1.4	20.5
37	12. 9. »	35	1.8	20.4
38	28. 10. »	23	2.0	20.4
39	26. 9. »	29	1.6	20.2
40	9. 4. »	27	1.4	20.0
41	10. 4. »	21	1.3	20.0
42	20. 11. »	24	2.0	19.8
43	4. 10. »	23	1.7	19.0
44	11. 9. »	29	1.9	18.5
45	11. 9. »	30	1.2	18.0
46	21. 11. »	24	1.1	17.8
47	12. 9. »	30	1.7	17.8
48	18. 9. »	35	1.7	17.5
49	5. 10. »	22	1.0	17.5
50	20. 7. »	26	1.5	16.0

Mean value 21.6
Standard deviation 2.40
Standard error of the mean.. 0.34

of the accommodation curve has been defined as the multiple of the rheobase reached at 1 sec. and obtained by graphical extrapolation of the initial rectilinear portion of the curve (see Fig.5.)

The average for the men is 21.2 ± 0.46, with the standard deviation 3.27. The average for the women is 21.6 ± 0.34, with the standard deviation 2.40. Thus in this material there is no statistically significant difference in the average values for men and women. The limits for the total number of cases were 16 and 31.2, whence there is some distortion to the left in the distribution of the values round the mean value. Values under 16 have never been observed by the author in normal conditions, and even 16 is doubtful. The female case in the table with the value 16 showed positive Trousseau and Chvostek signs, and thus had symptoms of a deficiency of ionized calcium. There is no correlation between the value of the rheobase and the slope.

3. Variations in accommodation in the same individual during a period of some length.

In order to obtain some idea as to how far the accommodation varies in the same individual during a period of some length, it

Table VII.

Variations in the initial accommodation slope during a period of some length.

Date.	Init. acc. slope determined on the ulnar nerve.		Date	Init. acc. slope determined at the motor point of *musc. flex. dig. subl.*		
	Case M-r	A-m		Case M-r	F-d.	K-n.
21. 1. 43.	28.0	24.5	16. 3. 42.	20.4	22.9	25.0
28. 1.	26.4	23.0	18. 3.	20.4	21.3	25.7
3. 2.	23.8	25.5	20. 3.	21.8	20.8	23.9
5. 2.	—	21.6	23. 3.	22.6	20.0	28.5
15. 2.	24.1	24.2	26. 3.	23.3	22.8	22.8
19. 2.	24.6	—	28. 3.	22.9	—	23.0
20. 2.	25.8	23.8	30. 3.	23.0	22.5	23.5
2. 3.	23.6	24.0	2. 4.	22.5	—	—
16. 3.	26.5	21.2	4. 4.	—	21.3	—
6. 4.	25.4	21.6	8. 4.	22.5	23.4	—
10. 4.	27.8	22.0	10. 4.	—	—	23.7
			11. 4.	23.8	21.8	—
			13. 4.	23.3	—	—
			14. 4.	—	23.5	—
			17. 4.	26.0	26.0	26.4
Standard deviation.	1.59	1.47		1.49	1.63	1.87

was repeatedly measured in the course of about one month, in tests on 4 healthy women of the age of 25; in 2 cases on the ulnar nerve, with a palpable minimal twitch in the tendon of *musc. flexor carpi ulnaris* as an index, and in 3 cases at the motor point of *musc. digitorum sublimis dig.* 4, with a minimal movement of the finger as an index.

The standard deviations were 1.59, 1.47, 1.49, 1.63 and 1.87, respectively. As the error inherent in the method itself is 1.09 (see p. 24), one can calculate the magnitude of the variations in these series which were due to changes in the accommodation of the nerve. They were found to be 1.4—1.5, or 6—8 per cent. of the mean value.

4. Comparison between the accommodation in short and long nerve fibres within the same nerve.

By stimulating the ulnar nerve at the elbow or the median nerve in *sulcus bicipitalis medialis*, long fibres to the hand muscles and shorter fibres to the muscles of the forearm can easily be stimulated under fairly identical conditions. The accommodation slope determined with a twitch in a hand muscle as an index thus refers to the longest fibres in the nerve, whilst the slope determined with a twitch in a forearm muscle as an index refers to the shorter fibres.

The results of the measurements of accommodation in short and long fibres of the ulnar and median nerves of healthy subjects are summarized in tables VIII and IX. For *musc. flexor carpi ulnaris, musc. flexor carpi radialis* and *musc. palmaris longus*, a minimal palpable twitch in the tendon of the respective muscles was taken as an index; for *musc. flexor digitorum sublimis et profundus, musc. opponens pollicis, musc. abductor pollicis brevis* and *musc. adductor pollicis*, a minimal movement of the finger; for *musc. interosseus dorsalis* 1, a directly observable minimal contraction.

The tables show that the *accommodation, measured as above described, is poorer in the long fibres of the ulnar and median nerves than in the short ones.* The difference is statistically significant. Futhermore, the breakdown of accommodation has been found to occur at somewhat lower relative current strengths in the long fibres than in the short ones.

Table VIII.

Comparison between the initial accommodation slope determined on short and long fibres in Nervus ulnaris, excited at elbow.

Case	Index: contraction of M. fl. carp. uln.		Index: contraction of M. add. poll.		Index: contraction of M. interossei.	
	R.	Slope I	R.	Slope II	R.	Slope III
1	1.0	22.5	0.8	20.0	0.85	18.6
2	1.0	22.5	0.9	19.8	0.09	20.4
3	1.5	28.5	1.5	21.0	1.5	22.5
4	1.6	25.8	1.6	19.2	1.6	13.2
5	1.1	18.0	1.1	16.0	1.1	14.8
6	1.3	21.1	1.3	17.4	1.3	16.5
7	1.6	24.5	1.6	17.4	1.3	17.2
8	1.3	18.6	1.0	15.1	1.0	16.5
9	1.3	22.0	1.5	18.0	1.5	16.0
10	0.8	19.6	0.75	15.1	0.9	13.6
11	1.4	22.6	1.1	19.4	1.1	19.2
12	1.5	30.5	1.4	22.6	1.8	24.2
13	1.5	24.0	1.2	21.7	1.1	19.5
14	1.3	28.5	1.1	23.5	1.2	23.0
15	1.1	23.5	0.9	18.6	1.1	16.2
16	1.4	18.4	1.3	13.2	1.2	14.5
17	1.4	22.0	1.1	16.0	1.4	19.0
18	1.4	24.5	1.6	19.8	1.6	23.0
19	0.7	21.7	0.75	18.0	0.8	18.2
20	1.1	20.5	1.1	15.0	1.1	15.0
Mean value.....		23.0		18.3		18.1
Mean difference				—4.7 (II—I)		—4.9 (III—I)
Standard error of mean difference				0.39		0.55

Table IX.

Comparison between the initial accommodation slope determined on short and long fibres in Nervus medianus, excited at the medial edge of musc. biceps brachii.

Case	Index: contraction of	R.	Slope I	Index: contraction of	R.	Slope II
1....	M. palm. long.	1.4	19.8	M. abd. poll. brev.	2.5	17
2....	M. flex. carp. rad.	1.7	23.1	M. abd. poll. brev.	1.6	17.6
3....	M. palm. long.	3.0	24	M. abd. poll. brev.	2.5	19
4....	M. flex. dig. subl.	2.3	23.6	M. op. poll.	2.4	17
5....	M. palm. long.	2.3	30	M. op. poll.	2.5	19
6....	M. flex. dig. subl.	1.3	23	M. op. poll.	1.8	17
7....	M. flex. carp. rad.	2.1	28.5	M. op. poll.	2.8	20.5
8....	M. flex. carp. rad.	1.3	26	M. op. poll.	1.3	20
9....	M. flex. carp. rad.	2.2	28	M. op. poll.	2.1	23
10....	M. flex. dig. prof.	3.0	30	M. op. poll.	3.0	23

Mean value 25.6 19.3
Mean difference ..... —6.3 (II—I)
Standard error of mean difference 0.68

5. Comparison between the accommodation in different nerves.

The results of the measurements of accommodation in different nerves of the same individual are summarized in table X. The ulnar nerve was stimulated somewhat proximally to the elbow, with a minimal palpable twitch in the tendon of *musc. flexor carpi ulnaris* as an index. The median nerve was stimulated at the medial edge of *biceps*. A minimal observable bending of the finger or palpable twitch in the tendon of *musc. flexor carpi radialis* or *musc. palmaris longus* was taken as an index. The radial nerve was stimulated on the outer side of the upper arm at the transition between the distal and central third part. An observable twitch in the tendon of *musc. extensor digitorum communis* or *musc. abductor pollicis longus* was taken as an index. *Nervus accessorius* was stimulated at, or somewhat behind, the rear edge of the sternocleidomastoid muscle, with a minimal observable contraction of the *trapezius* muscle as an index. Finally, *nervus facialis* was stimulated somewhat in front of the ear, with a minimal observable twitch in the *filtrum, ala nasi* or *mentum* as an index.

The experimental subjects referred to in table X were patients in the Neurological Clinical Department of the Serafimer Hospital, suffering from herniated *nucleus pulposus* in the lumbar region, lumbago of unknown etiology, neurasthenia and *encephalopathia traumatica*. Two of the cases were surgical, namely a distortion of the foot and a *status post appendicitidem*.

As will be seen from the table, the median nerve shows a somewhat better accommodation than the radial nerve, whilst the accommodation of the latter nerve is somewhat better than that of the ulnar nerve. Though the differences are small, they are statistically significant, or at any rate probable. There is no statistically significant difference between the accommodation as measured in the nerves of extensor and flexor muscles on the forearm. *Nervus accessorius* shows a somewhat better accommodation than that of the forearm nerves, but the difference relatively to *nervus medianus* has not been established with certainty. Finally, the *facial nerve* shows the best accommodation of all the nerves examined; but their curves often indicate a breakdown at a lower current intensity than might have been expected from

Table X.

The initial accommodation slope in different nerves of the same individual.

Case	N. ulnaris		N. medianus		N. radialis		N. accessorius		N. facialis	
	R.	Slope I	R.	Slope II	R.	Slope III	R.	Slope IV	R.	Slope V
1....	0.8	18.8	2.0	21.8	3.0	21.8	1.1	23.6	1.3	34.2
2....	1.2	22.6	3.5	23	3.0	23	0.7	29.4	2.0	38.5
3....	0.7	22.6	1.7	23.1	—	—	1.4	31.2	1.3	35
4....	2.0	26.2	2.0	28	2.5	25	1.6	28.5	0.9	29.5
5....	1.1	23.0	1.6	25	4.5	22.5	0.9	28	1.2	40.5
6....	2.5	18.8	2.1	22	2.1	20	1.3	23	1.2	31.2
7....	0.9	19.4	2.3	23.6	2.1	21	0.7	23	1.3	31.4
8....	0.9	26.8	0.9	28	1.7	25	0.9	32	—	—
9....	1.0	22.8	2.3	30	3.3	26	1.7	32	1.5	32.4
10....	1.9	27.8	2.7	31	2.4	31	0.8	36	1.6	40.5
11....	1.4	18.5	3.0	24	2.0	24	0.5	26	1.2	34
12....	0.9	22.3	1.4	23	2.8	21	0.4	23	0.8	30
13....	1.1	18.0	1.8	26.8	1.9	20.3	0.5	24	0.8	26
14....	0.7	22.5	2.1	28.5	3.3	23	1.3	26	0.9	29.8
15....	0.8	20.3	1.3	26	3.4	29	0.9	27	0.9	40
Mean value....		22.0		25.6		23.8		27.5		33.8
Mean difference				3.6 (II—I)		1.8 (III—I)		5.5 (IV—I)		12.1 (V—I)
Standard error of mean difference				0.67		0.77		0.62		1.21

Table XI.

The initial accommodation slope is different nerves on the lower leg.

Case	Index: contraction of			Index: contraction of M. peroneus long.		Index: contraction of M. gastrocnemius	
		R.	Slope I	R.	Slope II	R.	Slope III
1.....	Tib. ant.	1.5	18	1.4	19	1.7	19
2.....	Ext. dig. c.	1.4	21	1.8	20	1.0	27
3.....	Tib. ant.	1.4	22	1.0	21	2.5	21
4.....	Ext. d. c.	1.6	19	1.4	17	5.0	19
5.....	Tib. ant.	2.1	20	1.7	21	2.4	20
6.....	Tib. ant.	3.0	22	2.1	20	3.0	22
7.....	Ext. d. c.	1.1	16	1.1	17	2.7	20
8.....	Tib. ant.	3.0	23	1.1	21	3.0	23
9.....	Ext. h. l.	1.4	21	1.3	21	3.2	22
10.....	Ext. d. c.	1.6	20	1.6	21	4.2	18
Mean value			20.2		19.8		21.1
Mean difference			— 0.4 (II—I)				— 1.3 (II—III)
Standard error of mean difference			0.43				0.84

their steepness. It had been expected that the breakdown would take place at a relative current strength of about 4—5 times the rheobase: in about 50 per cent. of the cases, however, it occurs at a current strength of 2.5—3.5 times the rheobase.

The nerves of the leg muscles, as will be seen from table XI, show an accommodation with values corresponding to those of the forearm; here too no difference could be observed between the nerves of the extensor and flexor muscles.

The nerves of the extensor group of muscles and of *musc. peroneus longus* were stimulated at the *capitulum fibulae*, and the nerve of *musc. gastrocnemius* at the *fossa poplitea*. A minimal observable muscle contraction was taken as an index. The tests were made on patients without any signs of organic nerve lesions.

B. Motor Points.

I. Introduction.

After the investigations of REMAK (1855), v. ZIEMSSEN (1885), BOURGUIGNON (1923) and others, it has been a generally accepted view that stimulation of a motor point is stimulation of the nerve and thus merely an indirect stimulation of the muscle. Anatomically, the motor point is usually marked by the projection on the skin showing the place where the nerve enters, or branches off in the muscle. After BOURGUIGNON most of the numerous determinations of the chronaxie in man have been made at a motor point. The chief reason for this has been that, as the motor point is of relatively large extent, any shifting of the position of the electrode there causes a smaller change in the rheobase than at the little, sharply delimited nerve point.

Determination at the nerve itself, as carried out above, is therefore considered to be more laborious, as it is often found that the rheobase has changed during the course of the determination, so that it has to be repeated.

There is, however, a rather serious objection to stimulation at a motor point, namely the risk that the rheobase and the chronaxie may be determined on different organs, as the nerve and the muscle here lie in close touch with one another. That this actually occurs has been shown, for example, by PLATZ (1932), ACHELIS and BUESSOW (1932) and BUESSOW (1933). In fact, if

complete strength-duration curves are traced, they often have a break and are therefore composite curves, as demonstrated by LUCAS (1907 b) and RUSHTON (1930, 1931) on frogs. In such circumstances the ordinary mode of determination does not show the chronaxie either of the nerve or of the muscle, but some intermediate value. As will be shown, such composite curves are often obtained also in measurements of accommodation at a motor point, which, of course, complicates this mode of determination.

2. The accommodation curve.

If a muscle, e. g. *musc. biceps*, is stimulated beyond its motor point, a slow contraction in response to the stimulus is obtained even with currents of relatively small time-constants (about 30—50 msec.). After some probing, such a contraction can be elicited even by a current of rheobasic strength, as has already been observed by BOURGUIGNON and HUMBERT (1936). The accommodation curve for stimulation at such points is rectilinear and very flat, and apparently does not show any breakdown of the accommodation. In fact, even where the time constants of the stimulating currents amount to 300 msec., the linearity is maintained. Nor with currents up to 15—20 times the rheobasic strength is any cathodal closing tetanus observed in such cases, but merely a slow, though not persisting, contraction. The accommodation is much slower than that of the nerve and has been found to lie between 3.5 and 7.5, reckoned as "slope" (see Fig. 7).

On the stimulation of a motor point, curves of the same general form and gradient as those recorded on stimulation of a nerve are also frequently obtained. Only the value of the cathodal closing tetanus, expressed in the number of rheobases, is apparently higher and the closing tetanus, when elicited, of shorter duration. However, we also often find curves with a break, the initial steeper part of which has been determined with a distinct rapid contraction as an index, as in the stimulation of nerves, whilst a slower contraction has been taken as an index in determining their remaining, less steep, part (see Fig. 7).

The frequency of curves with a break differs considerably in different muscles and is perhaps determined by anatomical factors. They are rarely found on the stimulation of the long extensor and flexor muscles of the fingers, which have motor points extending

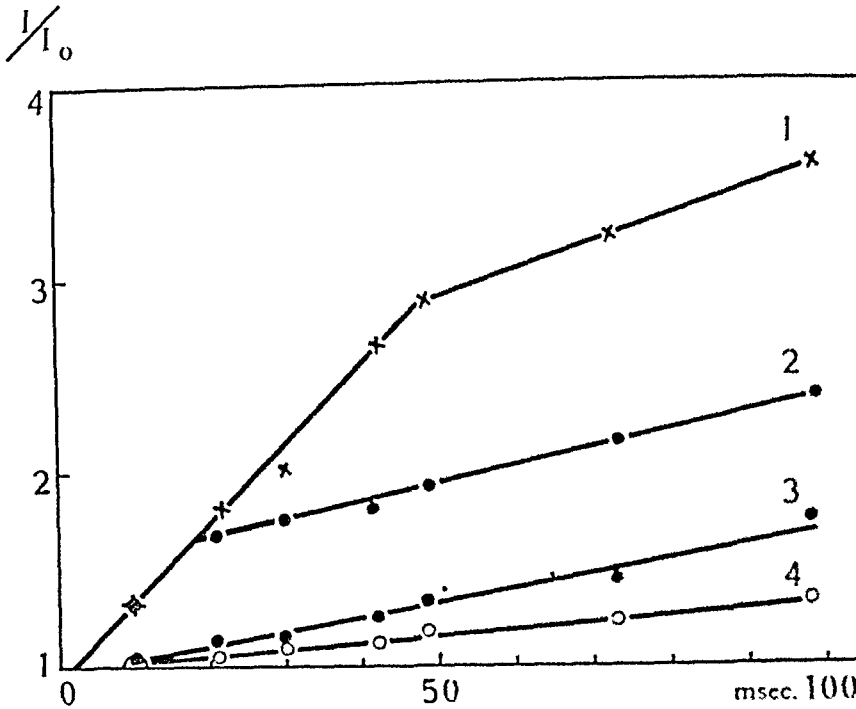


Fig. 7. Accommodation curves obtained by exciting *musc. biceps brachii*. Curve 1 and 2 by stimulation of the same motor point, with a minimal shifting of the electrode between the two determinations, showing the typical break. Initial slope: 41. Curve 3 stimulation outside the motor point, with a slow fascicular contraction as an index. Slope: 7.4. Curve 4 ditto, from another subject. Slope 3.7.

in the longitudinal direction of the muscles: this is probably due to the fact that the nerve lies on the muscle and follows it for some distance before it branches off.

On the other hand, on the stimulation of muscles such as *brachioradialis* or *flexor carpi ulnaris*, the muscles of the thigh, etc., curves with a break are almost always found, no matter what amount of care is taken to place the stimulating electrode exactly on the motor point. On stimulation of *musc. biceps*, breaks in the curve are sometimes found, sometimes not. Generally speaking, however, such breaks occur so frequently that it must be considered unwarrantable to base the measurement of the accommodation on merely a single point in the curve, without making sure that the index has been kept constant.

The occurrence of the above-mentioned typical breaks in the curve must be due to the fact that, during the course of the determination, different elements were stimulated, first, apparently, one

with a low threshold and rapid accommodation. Where the rising times are comparatively long and the current sufficiently strong, the second element is selectively stimulated: at the outset it evidently has a higher rheobase, but, owing to its poorer accommodation, it does not raise the threshold as speedily as the element with the more rapid accommodation. It should be noted that the slope of the more horizontal part of the curve with two phases is not a quantitative measure of the accommodation in the slow element (muscle?) from which it has been obtained. The rheobase in fact relates to the initial part of the curve and is thus too low: the accommodation therefore is lower than is shown by the curve, but otherwise somewhat indefinite.

It may be asked what is stimulated, the muscle or the nerve, when the slow contraction is observed. The most probable explanation is that we are concerned here with a direct stimulation of the muscle, seeing that its response to stimulation is quite different from that of the nerve: it shows a bad accommodation, besides which it lacks the cathodal closing tetanus and the breakdown of the accommodation. It is of interest to note in this connection that the idiomuscular response to an extraneous stimulus, as clinically tested with a percussion hammer, likewise manifests itself as a slow contraction in the stimulated part of the muscle. Also the degenerated muscle, in cases of peripheral nerve lesion, shows a bad accommodation or none at all (cf. the Introduction, p. 19) and responds to electric stimulation with a slow contraction. If we now assume, as is the generally accepted view, that the reaction of degeneration is the reaction of the denervated muscle, we shall gain further support for the supposition that the slow contraction, as above described, has been induced by direct stimulation of the muscle.

3. Limits of variations.

The accommodation was measured in tests on 100 healthy subjects (medical students or members of the hospital staff) at the motor point of *musc. flexor digitorum sublimis dig.* 3—4, with a minimal movement of the finger as an index. The material comprised 50 men and 50 women.

The average for the men was 23.5 ± 0.53, with the standard deviation 3.72, for the women 23.5 ± 0.39, with the standard

Table XII.

Normal values of the initial accommodation slope recorded for healthy male subjects. Determined at the motor point of musc. flexor dig. subl. 3 or 4. Index: minimal observed movement of finger.

Case	Date	Age	Rheobase	Initial Slope
1	14. 4. 1942	26	2.0	29.7
2	16. 4. »	37	1.9	29.1
3	30. 3. »	23	1.7	28.7
4	17. 4. »	30	1.8	28.4
5	14. 4. »	26	1.0	28.2
6	31. 3. »	27	1.8	28.2
7	15. 4. »	26	2.0	28.1
8	11. 4. »	27	1.1	27.6
9	10. 4. »	24	1.3	27.4
10	23. 3. »	23	1.8	27.4
11	26. 3. »	26	1.7	27.4
12	16. 4. »	36	1.4	26.9
13	30. 3. »	22	1.1	26.4
14	31. 3. »	31	1.9	26.3
15	2. 4. »	31	1.4	26.2
16	15. 4. »	28	1.8	26.2
17	8. 4. »	27	2.2	26.2
18	10. 4. »	29	0.9	24.6
19	30. 3. »	24	1.5	24.3
20	13. 4. »	19	3.5	24.2
21	11. 4. »	26	1.7	24.0
22	19. 3. »	24	2.1	24.0
23	30. 9. »	15	0.6	23.9
24	31. 3. »	22	1.4	23.8
25	31. 3. »	28	2.0	23.8
26	14. 4. »	27	0.9	23.7
27	15. 4. »	31	1.8	23.6
28	16. 4. »	33	2.3	22.7
29	18. 3. »	24	1.3	22.6
30	30. 9. »	35	2.5	22.5
31	27. 3. »	28	1.2	22.4
32	31. 3. »	25	1.1	22.3
33	11. 4. »	27	1.3	22.2
34	9. 4. »	28	1.2	21.8
35	9. 4. »	25	0.9	21.8
36	11. 4. »	24	1.0	21.6
37	28. 4. »	24	1.9	21.3
38	31. 3. »	17	1.8	21.0
39	13. 4. »	24	1.5	21.0
40	30. 9. »	22	0.9	21.0
41	28. 3. »	38	1.0	20.9
42	30. 3. »	23	1.6	20.7
43	30. 9. »	27	1.6	20.5
44	14. 4. »	25	2.1	20.0
45	18. 4. »	35	1.0	19.8
46	26. 3. »	22	2.0	19.6
47	11. 4. »	25	1.4	19.0
48	27. 3. »	23	1.4	18.0
49	10. 4. »	23	0.5	13.3
50	14. 4. »	26	0.4	12.6

Mean value	23.5
Standard deviation	3.72
Standard error of the mean	0.53

Table XIII.

Normal values of the initial accommodation slope recorded for healthy female subjects. Determined at the motor point of musc. flexor dig. subl. 3 or 4. Index: minimal observed movement of finger.

Case	Date	Age	Rheobase	Initial Slope
1	28. 3. 1942	21	2.0	29.1
2	14. 4. »	31	1.3	28.8
3	16. 4. »	22	1.6	28.8
4	16. 4. »	21	2.2	28.3
5	23. 3. »	22	1.5	27.9
6	15. 4. »	54	1.9	27.5
7	30. 3. »	24	2.1	27.4
8	27. 3. »	21	1.5	27.1
9	28. 3. »	30	2.1	26.7
10	9. 4. »	27	0.7	25.6
11	14. 4. »	30	1.0	25.0
12	10. 4. »	23	2.2	25.0
13	23. 3. »	28	2.4	25.0
14	27. 3. »	21	1.0	25.0
15	23. 3. »	21	1.6	24.9
16	9. 4. »	27	1.8	24.5
17	15. 4. »	22	1.4	24.4
18	16. 4. »	22	2.0	24.1
19	10. 4. »	27	2.3	24.0
20	2. 4. »	30	1.6	23.8
21	9. 4. »	21	1.9	23.8
22	13. 4. »	25	2.1	23.6
23	27. 3. »	26	1.9	23.5
24	28. 3. »	25	1.3	23.5
25	28. 3. »	32	2.2	23.5
26	30. 3. »	23	1.9	23.4
27	30. 3. »	21	2.0	23.3
28	23. 3. »	22	2.1	23.2
29	30. 3. »	24	1.3	23.1
30	30. 3. »	22	1.4	23.0
31	28. 3. »	21	1.3	22.9
32	23. 3. »	24	1.4	22.8
33	28. 3. »	27	2.6	22.3
34	16. 4. »	21	1.8	22.3
35	16. 3. »	20	1.7	22.0
36	23. 3. »	27	1.1	22.0
37	27. 3. »	30	2.3	21.6
38	28. 3. »	20	0.7	21.5
39	28. 3. »	31	1.0	21.1
40	20. 3. »	35	1.5	20.7
41	23. 3. »	40	1.5	20.7
42	27. 3. »	21	1.4	20.5
43	28. 3. »	22	1.3	20.0
44	27. 3. »	22	2.0	20.0
45	27. 3. »	20	1.0	19.8
46	27. 3. »	22	1.1	19.6
47	27. 3. »	20	1.0	19.6
48	27. 3. »	23	2.2	19.6
49	23. 3. »	25	1.4	19.0
50	2. 4. »	21	1.3	18.7

Mean value 23.5
Standard deviation 2.77
Standard error of the mean 0.39

deviation 2.77. The average for men and women is thus practically the same. It moreover exceeds the average values obtained on stimulation of the ulnar nerve by 2.3 ± 0.70 and 1.9 ± 0.55, respectively. This is a small, but statistically significant difference.

4. The accommodation at different motor points in the same individual.

The accommodation was measured in tests on 10 individuals (the same material as in table X) at different motor points: on a flexor muscle of the forearm, namely *musc. flexor digitorum sublimis*, with a minimal movement of the finger as an index; on an extensor muscle of the forearm, either *musc. extensor digitorum communis* or *musc. pollicis longus;* on the upper arm, *musc. biceps;* and on the trunk, *musc. obliquus externus abdominis,* with a minimal observable muscle contraction as an index.

Table XIV.

The initial accommodation slope determined at the motor point of different muscles in the arm and trunk of the same individual.

Case	Flexors of the fingers		Extensors of the fingers		Musc. biceps		Musc. obliquus externus abdominis	
	R.	Slope I	R.	Slope II	R.	Slope III	R.	Slope IV
1	0.9	21	1.6	22	1.2	31	2.5	33
2	2.5	23	3.0	23	0.8	24	3.5	39
3	1.6	21	1.7	25	0.9	32	2.0	33
4	3.4	24	1.7	21	2.5	32	1.8	32
5	1.5	20	1.1	22	0.7	34	1.1	30
6	0.8	29	1.7	34	2.4	23	3.0	33
7	1.8	25	1.9	21	1.7	24	1.0	32
8	1.3	24	3.0	26	1.6	30	3.3	35
9	1.5	22	3.0	22	1.2	54	2.2	40
10	1.0	20	1.7	21	0.8	29	2.1	32
Mean		22.9		23.7		31.3		33.9
Mean difference				0.8 (II—I)		8.4 (III—I)		11.0 (IV—I)
Standard error of mean difference				0.85		3.25		1.3

It appears from the table that there is no difference between the extensor and flexor muscles on the forearm. On the other hand, it is statistically established that *musc. obliquus externus abdominis* has a better accommodation than the muscles of the

forearm, and very probable that this is the case also with *musc. biceps* on the upper arm. The small muscles of the hand have shown an accommodation closely corresponding to that of the forearm, though possibly somewhat inferior. The accommodation at a motor point in *musc. triceps*, *deltoideus*, *trapezius* and *rectus abdominis*, in those cases where it has been investigated, has been found to be similar to that of *musc. obliquus externus abdominis.*

To sum up, it may thus be stated that the accommodation, measured at a motor point, is about 30 to 40 per cent. better in the muscles of the trunk, shoulder-girdle and upper arm than in those of the forearm and hand. No difference between the extensors and flexors has been observed.

C. Sensory Nerves.

1. Introduction.

It has been known since ERB (1872) and HOFFMANN (1888) that, in the clinical test, the motor and sensory nerves behave in exactly the same way, and that in the sensory nerve a "*Katoddauerregung*" corresponds to the cathodal closing tetanus. The only quantitative difference observed is that the thresholds for stimulation of a sensory nerve are somewhat lower than for a motor nerve. EBBECKE (1922, 1924) made a thorough study of the reaction of a sensory nerve to a galvanic current and especially of its correspondence to the cathodal closing tetanus; this reaction, which, however, had previously been noted by v. FREY (1896), he termed "*Nervenschwirren*". In fact, on the stimulation of a sensory or mixed nerve with a current of low strength, a short, but distinct, shock is felt in the territory of the nerve. When stronger currents — according to EBBECKE, about six times as strong as the rheobase — are applied, they produce an intermittent, slightly vibrating, buzzing sensation, which soon dies away. This is just what he means by "*Nervenschwirren*", v. FREY's "oscillating sensation". On the application of a still stronger current this sensation increases in intensity and duration.

To v. FREY (1896) must be given credit for having shown that "*Nervenschwirren*" is identical with a form of paresthesia clinically known as formication, tingling, etc. EBBECKE (1922) has enumerated various pathological conditions in which it occurs, such as sciatica, shot wounds affecting a nerve, tabes and disseminated

sclerosis. He also mentions some experimental methods of inducing this sensation: mechanically, by a direct blow on the nerve, in the palms by clapping them against one another, in the lips by rubbing a coarse towel against them. According to him, "*Nervenschwirren*" occurs also transiently on anesthetization with novocaine, before local insensibility has set in. He adds that "*Nervenschwirren*" is identical with the familiar feeling of "*Eingeschlafensein*", which occurs during the initial stage of pressure paralysis or during recovery from such paralysis.

The last-mentioned statement is certainly incorrect, seeing that — as will be shown further on — paresthesias before and after ischemia are not of identical nature.

Though the identity of "*Nervenschwirren*" with the paresthesias occurring in cases of tabes and sciatica seems to require verification, "*Nervenschwirren*" is nevertheless a typical and well-defined form of paresthesia.

By stimulating with more or less rapidly rising currents, produced by manually varying a sliding resistance, EBBECKE ascertained that the sensory nerve also had a capacity for accomodation.

2. General results.

In taking accommodation curves of sensory nerve fibres, sufficient time must be allowed to elapse between each determination of threshold, seeing that — as EBBECKE (1922) has pointed out — the excitability is modified for several seconds after the cessation of the stimulus. Rapidly rising currents produce the sensation of a short, distinct shock, the threshold of which can be easily perceived. The response to somewhat more slowly rising currents (30—40 msec.) is more prolonged and blurred. To still more slowly rising currents the response is a softly vibrating, discontinuous sensation on the surface of the skin. Though the experimental subject may at first find some difficulty in indicating the threshold of excitation with such currents, he will be able to do so after some practice, with a considerable degree of accuracy. The accommodation curves of sensory nerves can therefore be determined with about the same precision as those of motor nerves.

A typical accommodation curve, taken after stimulation of the ulnar nerve at the elbow, with the sensation of a shock passing into a feeling of vibration at the hypothenar eminence as an index of the excitatory effect, is shown in Fig. 8.

It will be seen that the curve, though fairly steep in its initial part, bends away from the very outset, so that, at about the point marking a time-constant of 50 msec., it definitively takes a nearly horizontal course. In view of this configuration, the accommodation — as measured by the initial slope of the curve — is not well defined. It is possible, however, to obtain from the curve a quantitative measure of the breakdown of accommodation. As the breakdown here is almost complete, it can be computed more accurately than is possible in the case of the motor nerve.

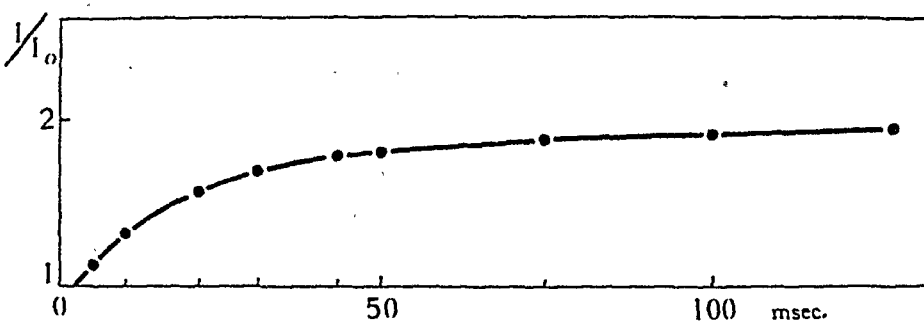


Fig. 8. Accommodation curve for tactile fibres in *nervus ulnaris*. Rheobase 0.4 m/Amp. Breakdown 1.85 times the rheobasic strength.

In order to obtain a uniform basis of comparison, the occurrence of the breakdown has been reckoned in table XV on the basis of the relative strength of the threshold of a current with a time-constant of 70 msec. At this point the curve has invariably taken its final course. The actual breakdown usually occurs somewhat earlier, but, in view of the flattened course of this part of the curve, the value, at a time constant of 70 msec., will be merely a few per cent. too high. The results of the stimulation of pure cutaneous nerves will be similar to those given in the table. The ulnar nerve was selected in order to obtain simultaneously a basis of comparison between the rheobase of sensory and motor fibres under fairly identical conditions.

As shown by the table, the figure for the breakdown of accommodation varies within fairly narrow limits. It lies at a relative current strength of 1.5—2 times the rheobase, whereas the corresponding figure for motor nerves is 2.5—5 times the rheobase. The mean value (1.85) accords well with that found with his electrical index by SKOGLUND (1942), namely 1.5, in the coarser fibres of cat nerves. The threshold for the sensory fibres is 42 per cent. lower than for the motor. Taking the nerve action potential as an

index, ERLANGER and BLAIR (1938), in experiments on frog nerve, found the corresponding value to be 20 per cent., whilst SKOGLUND (1942), in cat nerve fibres probably coming from the muscle proprioceptives, found it to be 24 per cent.

Table XV.

Rheobase and breakdown of accommodation in tactile fibres, and rheobase in motor fibres, of ulnar nerve.

Case	Date	Age	Tactile fibres		Motor fibres
			Rheobase	Breakdown	Rheobase
1	21. 11. 1942	31	0.32	1.80	0.7
2	23. 11. »	30	0.45	1.72	0.75
3	24. 11. »	26	0.60	1.87	0.95
4	24. 11. »	27	0.95	1.98	2.40
5	24. 11. »	35	0.45	1.94	0.90
6	24. 11. »	24	0.70	1.77	1.35
7	24. 11. »	29	0.85	1.93	1.16
8	25. 11. »	21	1.10	2.00	1.75
9	25. 11. »	26	0.73	1.87	1.35
10	25. 11. »	29	0.54	1.80	1.15
11	27. 11. »	27	0.45	1.84	0.85
12	27. 11. »	33	0.60	1.90	1.1
13	27. 11. »	26	0.85	1.90	1.2
14	28. 11. »	42	1.60	1.50	2.2
15	28. 11. »	23	1.15	1.90	2.0
		Mean	0.76	1.85	1.32

Bearing in mind that the value for the breakdown of accommodation is almost identical with that of the cathodal closing tetanus, it may be stated, in brief, that the *sensory nerve fibres* thus examined have, in the *normal state*, the same features as those which ERB, as far back as 1874, found to be characteristic of *the motor nerve in tetany*, namely a low rheobase, and, both absolutely and relatively, a low threshold for the cathodal closing tetanus. The sensory nerve fibres thus show a greater tendency towards iterativeness than the motor fibres (cf. SKOGLUND, 1942; GRANIT and SKOGLUND, 1943); the latter, however, can be made to show this tendency in equal degree by reduction of the ionized calcium.

The duration of the iterativity depends on the strength of the stimulating current. In Fig. 9 the duration of the vibratory sensation has been plotted against the stimulus strength. The ulnar nerve was stimulated at the elbow. The sensation is intense for the first few moments, but gradually dies away. As it is impossible to

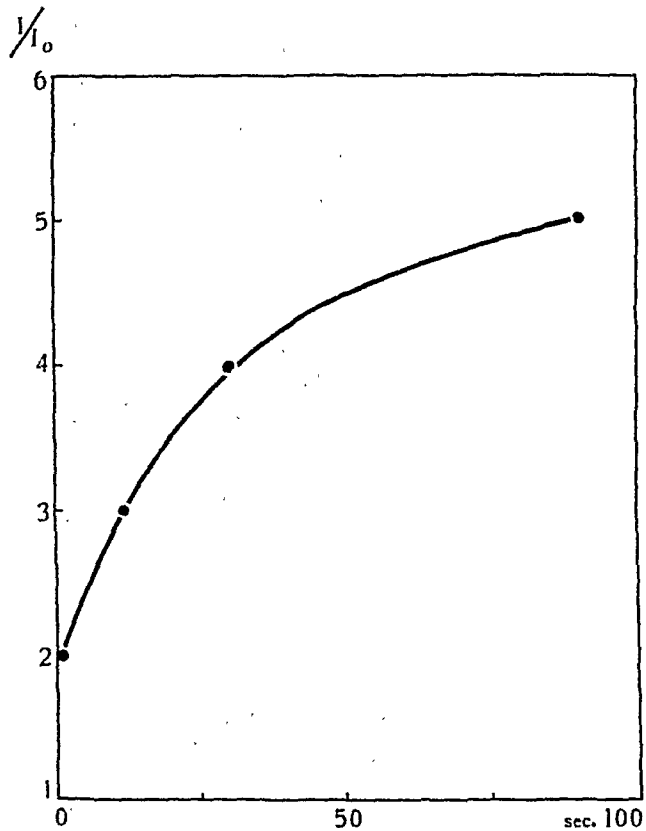


Fig. 9. Duration of vibrating sensation in sec., as function of stimulating current strength, expressed in multiples of rheobasic strength.

state exactly where the sensation, which eventually becomes quite faint, has actually ceased, the values in Fig. 9 are merely approximative.

An evidently prolonged cathodal closing sensation, indicative of repetitive discharges, and thus corresponding to the cathodal closing tetanus in the motor nerve, is already felt at twice the rheobasic strength; this might indeed have been expected from the fact that the breakdown of accommodation occurs at about that current strength. The values given by EBBECKE (1922), namely about six times the rheobasic strength, have been found to be unduly high.

The sensory fibres studied here have a low threshold and must therefore be of large calibre. The localization of the vibratory sensation on the surface of the skin and its occurrence also on the stimulation of purely sensory nerves rule out the supposition that

we may be concerned here with fibres from the muscle proprioceptives, which likewise have a relatively large diameter (SHERRINGTON 1894). In all probability therefore they are tactile fibres. v. FREY, too, localized the *"Nervenschwirren"* in the tactile elements (see above). The sensation is likewise of tactile character, though it does not correspond to that felt on adequate stimulation of the skin. This supposition is also borne out by the fact that the sensation of light touch is reduced in the paresthetic region, so that the experimental subject often spontaneously remarks that he feels numbness there. In fact, the sensation is evidently identical with that produced by HEINBECKER, BISHOP and O'LEARY (1933) as the first response to *repeated* stimulation when the current strength is gradually increased. These authors interpreted it as tactile. The paresthesia corresponding to this sensation must therefore likewise be a tactile paraesthesia.

D. Summary and Conclusions, Section III.

1. The accommodation curve determined on a motor nerve consists of an initial rectilinear portion, the slope of which for slowly rising currents begins to diminish gradually and terminates in a more horizontal portion at a current strength of 2.5—5 times the rheobase. A breakdown of accommodation thus occurs at that strength, and consequently the nerve response to the stimulating current is repetitive and the muscle response a tetanus. Breakdown of accommodation and "cathodal closing tetanus" are closely related. The latter likewise occurs at 2.5—5 times the rheobase. As a rule, the greater the initial slope of the curve, the greater the current strength necessary for breakdown of accommodation.

The average value for the initial slope of the accommodation curve, given in number of rheobases by extrapolating the linear portion to 1 sec., is for healthy men 21.2 ± 0.46, with a standard deviation of 3.72. The ulnar nerve was stimulated and the index was a minimal contraction in the tendon of *musc. flexor carp. uln.* For women the corresponding value was 21.6 ± 0.34 and the standard deviation 2.40.

In the course of about one month the value for the initial slope of the same subject varies by 6—8 per cent. of the average.

In the median and ulnar nerves accommodation, measured as

initial slope, is greater in the short fibres of the forearm muscles than in the long fibres of the small muscles of the palm. The difference is small, but statistically significant. It is probably a real difference between the fibres concerned, but may be apparent and merely due to the use of different muscles as an index in the measurements. As a rule, accommodation varies relatively little in different nerve fibres of the same subject. An exception is the facial nerve, which has about 30 per cent. greater accommodation than the arm and leg nerves. There is no difference in accommodation between extensors and flexors.

2. If the accommodation curve is determined at a *motor point*, it is often found to be separated into two portions by an angle, indicating that in the course of a single experiment different elements are being stimulated. One of them is characterized by a curve which is clearly identical with the accommodation curve as determined at a *nerve point*, and in this case the muscle contraction, elicited by the stimulus, is rapid. The other element has a badly developed accommodation and the muscle contraction is slower. There are reasons for presuming that in the latter case muscles have been directly stimulated.

Owing to the appearance of angles in the curve, determinations at motor points are unsatisfactory.

At motor points the accommodation is 30—40 per cent. greater in the muscles of the body and the upper arm than in the muscles below the level of the elbow.

With *musc. flexor digitorum sublimis* as an index, the limits of variation in healthy men are 23.5 ± 0.53, with the standard deviation 3.72, for women 23.5 ± 0.39, with the standard deviation 2.77, all values being calculated as initial slope.

3. Sensory fibres. The slope of the accommodation curve for fibres transmitting light touch successively diminishes from the outset, so that the accommodation can scarcely be defined, as in the case of motor fibres, by the initial slope of the curve. The whole curve then flattens out and the breakdown of accommodation takes place at 1.5—2 times the rheobasic strength. Thus the threshold for prolonged repetitive discharges is lower than in motor fibres. The rheobase too is lower in tactile than in motor fibres.

IV. Changes in nerve accommodation under the influence of ischemia; including notes on the nervous mechanism of fasciculations and on two different forms of paresthesia.

Historical section.

1. The disturbances.

The nervous disturbances in man due to circulatory arrest are closely connected with the changes in the affected nerves in response to electric stimuli. Deeper knowledge of these changes will throw some light on those disturbances. Conversely, the changes in the stimulated nerves will be more easily understood if dealt with in close connection with such disturbances and not treated independently of the latter, as has usually been the case. It seems desirable therefore to begin by giving a brief description of the nervous disturbances in ischemia. For a more detailed treatment of the subject, the reader is referred to the studies of LEWIS, PICKERING and ROTHSCHILD (1931) and ZOTTERMAN (1933).

If the blood supply to an arm is arrested by a pneumatic cuff round the upper arm, distended above the systolic pressure, a *centripetally* proceeding paralysis will develop, as shown by LEWIS, PICKERING and ROTHSCHILD (1931). The longest nerve fibres are thus the most susceptible to ischemia. The appreciation of touch, position, pressure and vibration is first paralyzed. As regards touch, this phenomenon is first noticeable as a feeling of numbness; somewhat later it takes the form of complete anesthesia, extending from the finger-tips up towards the place of compression. Then ensues loss of motor power, followed by loss of perception of cold, heat, rapidly conducted and slowly conducted pain, in the order of enumeration. All these faculties are paralyzed chiefly centripetally except motor power, the loss of which does not pro-

ceed uniformly round the arm, the extensors being paralyzed before the flexors.

The *differential loss* of faculties was first observed by HERZEN (1886). Confirmed by FABRITIUS and v. BERMAN (1913), it has been more thoroughly investigated by LEWIS, PICKERING and ROTHSCHILD (1931), ZOTTERMAN (1933), LEWIS and POCHIN (1938). The tactile sensation is transmitted by fast fibres of the A group and the temperature sensation by δ fibres of the A group (ZOTTERMAN, 1936). After the investigations of ADRIAN (1931), CLARK, HUGHES and GASSER (1935), ZOTTERMAN (1939) and PFAFFMAN (1939), it may be regarded as established that pain is conducted both by δ fibres and fibres of the C class. In man ZOTTERMAN (1933) as well as LEWIS and POCHIN (1938) have shown that the delayed second-pain response is conducted by C fibres.

The apparent explanation of the above-mentioned order of involvement of the various sensory qualities is thus that the coarser the fibres, the more readily are they paralyzed by the ischemia. This view was advanced by LEWIS and his associates (1931), in analogy with the observation made by ERLANGER and GASSER (1929) that the coarsest fibres in frog nerve are the most susceptible to pressure.

LEWIS and associates have also found that the more centrally the pneumatic cuff is placed, the more rapidly does paralysis ensue. Moreover, since the paralysis proceeds centripetally, the longest fibres — as has been previously indicated — are the most sensitive. From this it might be inferred that the longer the stretch of fibre exposed to ischemia, the greater the rapidity with which paralysis will set in.

This, however, is not the real explanation. In point of fact, the paralysis develops in the same manner, and with as great rapidity, if merely a limited stretch of the nerve is rendered ischemic by a compression which does not affect the circulation in that part of the nerve situated distally thereto. This can also be shown by placing and distending another pneumatic cuff some little distance distally to the first one, as soon as numbness has commenced in the finger-tips, and afterwards removing the proximal cuff. It will be found that, when the blood-flow is thus restored to the proximal part of the nerve, the numbness will rapidly clear away, despite the fact that the access of the blood to the periphery is blocked by the distal cuff. But, if one waits till the

numbness has spread and places the last-mentioned cuff near the original one, the numbness will *not* clear away.

These and other experiments of LEWIS and associates, which need not be reported here, show that *paralysis is produced first and foremost in the most proximal part of the ischemic nerve, by a block which spreads centrifugally, and that this block most affects those fibres which extend furthest out towards the periphery.*

The fact that the block spreads beyond the spot subjected to compression is one indication, amongst others, that it is the arrest of the blood-flow as such and not the pressure which is the principal cause of the paralysis. LEWIS and co-workers also noted that the block ensued with greater rapidity if the nerve was warmed, owing, as they supposed, to the increased rate of metabolism in the nerve. When the blood-flow is released, motor power and sensibility are quickly restored, but in inverse order to that of involvement. Thus anesthesia disappears last in the finger-tips, and the muscles of the hand recover their motor power last. Though this takes place in 25—50 seconds after an ischemia which has lasted 35—40 minutes, hours elapse before the complete recovery of the nerve. For, if the blood-flow is re-arrested within the last-mentioned space of time, the paralyses develop more rapidly than if the arm had been quite fresh.

Sensory phenomena of excitability. A few minutes after the commencement of the ischemia, paresthesia sets in, chiefly in the hand. Though an extremely characteristic phenomenon, it has received but little attention. When observed, it has evidently been identified with the paresthesia ensuing on the release of the blood-flow after a sufficiently lengthy compression, the same designations having been applied to both. BOURGUIGNON (1923) calls it *"fourmillion"*, EBBECKE (1922) *"Nervenschwirren"*.

About 60 seconds after the decompression, the pricking and stinging sensation known as "tingling" sets in. It has been thoroughly studied by LEWIS and co-workers (1931) as well as by ZOTTERMAN (1933). Its duration, intensity and propagation depend on the duration of the preceding ischemia. As a rule, it takes at least four or five minutes to produce it. It is most easily elicited at the periphery. After more protracted ischemia, its intensity and area of distribution are increased, so that it spreads centripetally up the arm.

Though apparently located in the skin, namely in the area of distribution of the ischemic nerve, this sensation nevertheless

has its origin in the most proximal part of the nerve, being the part most affected by the ischemia. This can be easily demonstrated by placing and distending a second pneumatic cuff distally to the original cuff. On the removal of the latter, tingling ensues, despite the arrest of blood-flow to the periphery. Moreover, the further proximally the cuff is placed, the easier is it to produce tingling. Hence it is most readily elicited in the most proximal part of the longest nerve fibres.

Though tingling thus does not actually originate at the periphery, it is considerably affected by impulses from the periphery within the area where it is felt. *Tactile* stimuli tend to increase its intensity. According to LEWIS and co-workers, this latter effect will fail to manifest itself where the ischemia has lasted so long that touch perception at the spot in question has been suspended, even if the perception of pain still persists. Thus excitation by pain or heat will not produce any such affect. From these observations LEWIS and associates deduce the inference that tingling originates in tactile fibres. ZOTTERMAN (1933), on the other hand, considers it probable that tingling originates in finer fibres than the tactile ones, for one reason because "the pricking sensation of tingling is felt as an acute pricking pain, as when a knife is stuck into a carbuncle". This, he thinks, indicates that the sensation is produced by nerve fibres conducting pain.

Motor excitability phenomena resulting from ischemia. In cases of reduced ionized calcium in the blood, motor phenomena, such as the Trousseau sign, are known to occur during ischemia. They will not, however, be discussed in this place.

It is also a known, though little noticed, fact that the Trousseau sign may occur after decompression. PHLEBS (1913) and NOTHMAN (1937), among others, have reported that, in rare cases, they have observed a typical Trousseau sign after ischemia, though not during its course.

Transitions from the Trousseau sign to fibrillar-fascicular twitches in the hand muscles are not unusual. This brings us to the fibrillar twitches described by REID (1931). He observed that after decompression fibrillar twitches, increasing in frequency on movement, normally occurred, especially in the small muscles of the hand. He considered that they were due to an excitatory affect of the reflowing blood directly on the muscle.

LEWIS and associates have described a phenomenon which they term "pseudo-cramp", a sensation of cramp in the hand with no —

or very slight — objective symptoms. They considered it to be due to an excitation of the muscle-spindle apparatus. It is thus a sensory phenomenon.

2. Changes in electric excitability.

Since the investigations of R. GEIGEL (1893), it is well known that the thresholds of the opening contraction are subject to considerable changes under the action of ischemia. They are, however, beyond the scope of the present study. The nature of these contractions is still too obscure to be brought into connection with the various motor and sensory disturbances which occur.

From experiments on frog nerve *in vitro* it is known that the rheobase increases, after a transitory initial lowering, under the action of asphyxia [THÖRNER (1924), HEINBECKER (1929) and others]. The same observation applies to excised cat nerve *in vitro*, according to LEHMANN (1937 b), whose thorough study of the effect of asphyxia on the A nerve fibres will now be reported. It should be noted, however, that, without further investigation, his results cannot be compared with those obtained during ischemia on human nerves *in situ*.

LEHMANN exposes the nerve to a mixture of nitrogen and CO_2. During the first 6 minutes the threshold is lowered, in order afterwards to rise rapidly until, after the lapse of about 30 minutes, the nerve ceases to respond to the stimulus. During the stage of low threshold, spontaneous firing, the extent of which depends on how low the threshold falls, occurs in the peroneal nerve. In the saphenous nerve, on the other hand, little or no spontaneous activity is observed. However, nerves of saphenous type can be made to react in the same way as those of the peroneal type by reducing the ionized calcium in the nerve. Conversely, nerves of the peroneal type can be caused to react like the saphenous nerve by the administration of ionized calcium. On the readmission of oxygen, the threshold falls rapidly to near the original value, but then again rises, and remains raised, for about 50 minutes.

No spontaneous activity is mentioned by LEHMANN as occurring during the recovery stage. He finds that exactly the same changes in threshold and after-potentials, as well as the spontaneous activity in the early asphyxia phase, can be produced by shifting pH towards the alkaline side or reducing the calcium ions. The threshold curve in the recovery phase also agrees with that obtained when an alkaline nerve is restored to normal. On the basis of these findings, he discusses the possibility of reducing all the changes observed during and after asphyxia merely to changes in pH or the amount of calcium ions.

A lowered threshold during ischemia in animal nerves *in situ* has been observed by MACCALLUM (1913), PATON, FINDLAY and WATSON (1916), and MORRIS (1922). In man, BOURGUIGNON and LAUGIER (1923) have studied the variations in the rheobase and the chronaxie, during stasis and ischemia, on the radial nerve and at the motor point of *musc.*

indicis proprius. The chronaxie showed the same variations at the nerve and its motor point, whereas the rheobase varied somewhat less at the motor point. The rheobase rises from the outset in the nerve itself until it ceases to respond to the stimulus: such non-excitability, on the other hand, is never found at a motor point. They interpreted this as "*une véritable curarisation*", that is, as an indirectly suspended, but directly retained, excitability of the muscle.

However, as LEWIS and co-workers have shown, this conclusion, plausible as it sounds, is incorrect. It is merely the proximal part of the nerve that is blocked and loses its excitability. If the electrode is placed further distally, excitability will be maintained also in the nerve itself. After the ischemia the threshold is again lowered, though not as far as the original value, but then rises again and is maintained above that value for an hour or so. The chronaxie is shortened during anoxemia, but is afterwards considerably lengthened and then gradually returns to normal. During the stage with a lengthened chronaxie the muscle contraction, in response to direct or indirect stimuli, is slow, "*analogue à celui de la réaction de dégénérescence*".

THOMPSON and KIMBALL (1936) studied the threshold of the sensory response to alternating current during ischemia in a nerve at the wrist, and likewise found an initial lowering of the rheobase.

In regard to the variations of accommodation during ischemia, no investigations, either in human or in mammalian nerve, are available. In frog nerve LIESSE (1938) found that the accommodation, measured at a point on the accommodation curve, became more rapid during ischemia and changed irrespectively of the chronaxie, which was little or not at all affected.

Experimental section.

Technique.

All the experiments were made on the arm; on the ulnar nerve. That nerve was selected because it affords technical facilities for studying the changes in excitability simultaneously at different points. With the same index, e.g. a minimal twitch in *musc. inteross. dors.* 1, and thus on the same nerve fibres, one can determine the excitability at the axilla, elbow or wrist. Moreover, the changes in excitability in the long fibres of the hand can be compared with the shorter fibres of *musc. flexor carpi ulnaris* by taking as an index a twitch, now in the hand muscle, now in *flexor carpi ulnaris*. The rheobases for these two indices always lie close to one another, which facilitates the determinations of thresholds: otherwise a concurrent marked twitch in the one muscle would interfere with the records taken of the other.

The determinations were made in the following manner. An electrode was fixed at the elbow (in certain cases also at the wrist, if the excitability at that point was to be investigated). An ordinary pneumatic cuff (10 cm. in width) was then placed as far up the arm as possible, in order that, on its distention, the position of the electrode should be affected in the least possible degree. Before the actual test, the cuff was provisionally distended and repeated determinations were made for a few minutes, in order to ensure that the rheobase was kept constant throughout. The cuff was then definitively distended to about 200 mm Hg, well above the systolic pressure, to arrest the blood-flow. In order to avoid cooling, the arm was kept in water heated to 37° C., or was wrapped in cotton-wool warmed with lamps, so that the same temperature was maintained under it. However, similar results were obtained when the arm had not been warmed: the warming therefore was considered unnecessary, except for a control test in each series.

Results.

1. The Rheobase.

Fig. 10 shows typical threshold curves taken after stimulation of the ulnar nerve at the elbow. The determinations were made simultaneously on the long fibres of the hand, with a minimal movement of the thumb as an index (the same results were obtained with a twitch in the *interosseus*) and on the shorter fibres of *musc. flexor carpi ulnaris*, with a minimal, palpable twitch in the tendon of that muscle as an index.

In correspondence with the previously reported investigations of nerve *in vivo* and of excised animal nerve *in vitro*, the threshold is lowered, that is, the excitability is increased, during the first 10 to 20 minutes. The threshold then rises above the original value: first slowly, then as a rule with increasing rapidity; complete lack of response to the stimulus was, however, attained only in one case out of ten, during the lengthy time, 30 minutes, for which the ischemia lasted. As soon as the blood-flow is released, the threshold is again rapidly lowered: after 30 minutes ischemia to about 50—70 per cent. below the original value.

In this respect my curves deviate from the rheobase curves published by Bourguignon and Laugier (1923), where the threshold does not fall below the initial value. The reason for this disparity is not understood. After a shorter ischemia, of 10 to 15 minutes, however, no, or very slight, changes in threshold before or after the compression can be observed in the short fibres. During this second stage of increase of excitability (lowering of threshold) the slow muscle contraction described by Bourguignon and Laugier is observed in response to the stimulus. After a rough exponential curve, the threshold then rises again above the initial value, where it is maintained for a considerable time, depending on the duration of the preceding ischemia.

As might be expected from the fact that the paralysis in is-

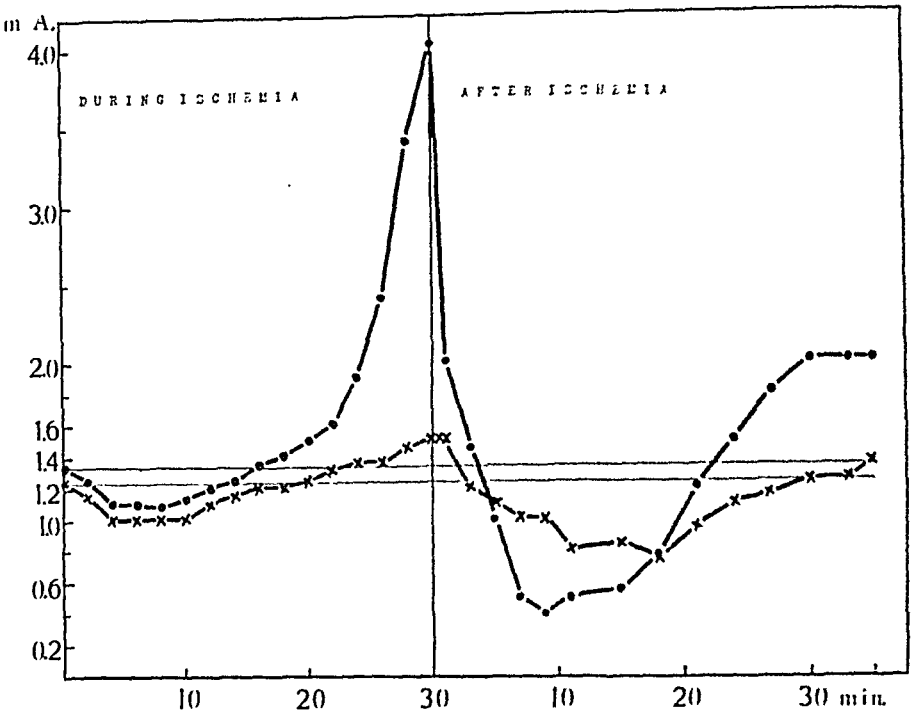


Fig. 10. Threshold curves from long and short fibres in the ulnar nerve at the elbow during and after ischemia. ●——●——● Thresholds for long fibres to *musc. add. poll.* ×——×——× Thresholds for short fibres to *musc. flexor carpi ulnaris.*

chemia is centripetal, or, in other words, that *the longest fibres are most easily affected, the changes in threshold are most marked in those fibres*, as is shown by the Figure. Thus, during ischemia, the threshold for the fibres of the hand not only rises more rapidly than for those of the forearm, but also drops lower: hence the increase in excitability after the decompression is most pronounced there. These facts are easily observed, in that the contraction in the hand muscles in response to the stimulus is at first about as marked as in the *flexor carpi ulnaris*, whereas after ischemia for 25 to 30 minutes a current just strong enough to elicit a twitch in the hand produces a violent contraction in the forearm. After decompression the reverse happens, though the disparity is less marked.

2. The accommodation curve.

Accommodation curves were taken during the course of ischemia and after. Such a series of curves, selected as typical examples

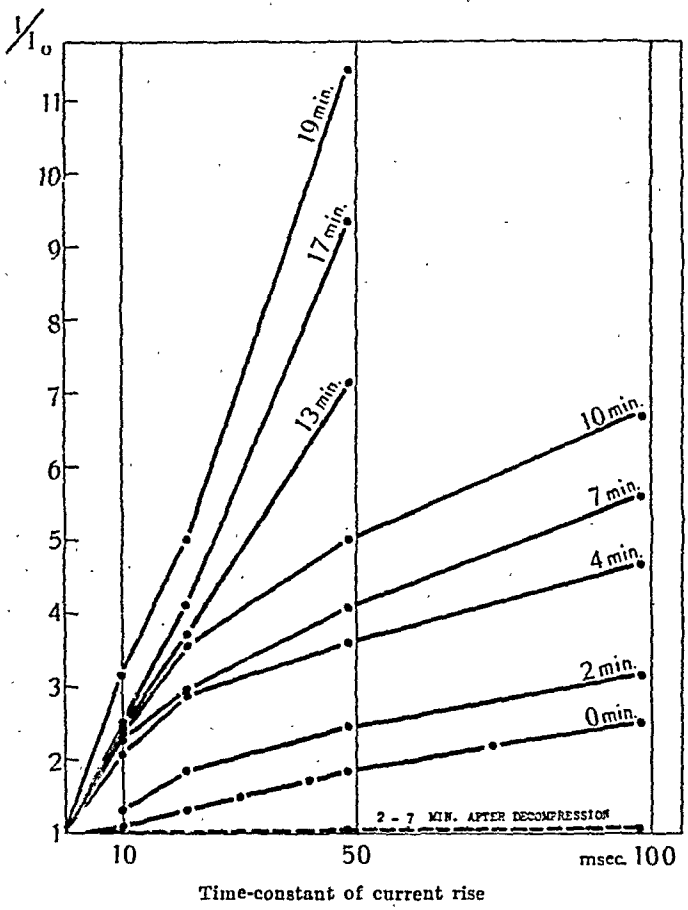


Fig. 11. Changes in the accommodation curve during and after ischemia of the ulnar nerve at the elbow in fibres to *musc. inteross. dors. 1*

among 10 tests showing great uniformity, is reproduced in fig. 11. In these tests the stimulating electrode was placed at the elbow, and a minimal twitch in *musc. interosseus dors.* 1 was taken as an index.

As will be seen from the figure, the curves for accommodation during ischemia tend to increase in steepness: this tendency, however, is most pronounced in the initial part of the curve, but afterwards becomes less and less marked. The curve thus deviates from its linear initial part and takes the form of a bend, with a gradually diminishing slope. The bend is real and cannot be attributed simply to the measurement technique, as is evident from the following considerations. The excitability of the nerve, of course, changes with comparative rapidity, and it takes one or two minutes to trace a curve. It is therefore obvious that, if the determinations

had been made with successively diminishing time constants, the thresholds of the more rapidly rising currents would in the meantime have risen somewhat, and would thus have been computed too high relatively to those first determined. In such circumstances we should have obtained upwardly convex curves. Here, however, the determination was made in the reverse direction, whence the curves would have been slightly concave upwards if the change in threshold during the course of the measurement had been a factor of any importance.

In tests on man, this type of curve must be regarded as pathological and has never been observed under normal conditions. SKOGLUND (1942) found it occasionally in cats and regarded it as a normal variation. It is, however, conceivable that, in this case too, it may have been due to the disturbance of the circulation caused by the preparation. Similar deviations from the normal linearity of accommodation curves have been observed, for example, by KAHLSON and v. WERZ (1936), in experiments on frog nerve.

As the figure indicates, accommodation curves of the type which, in regard to excised frog nerve, is considered to be quite *normal*, are obtained during the course of the ischemia. Thus in the asphyctic human nerve there is no "breakdown" of accommodation nor cathodal closing tetanus: it therefore shows the "minimal current gradient" defined by LUCAS (1907 a), during which no stimulus is effective, even if it has a strength of 8 times the rheobase. Indeed, after ischemia lasting 30 minutes, stimulation with a current with a rising time of 100 msec. and a strength of up to 20 times the rheobase has been found to be quite ineffective. It therefore seems necessary to verify these findings by control tests on the nerves of cold-blooded animals in which the circulation properly functions and not immediately to accept as physiological the results obtained with excised nerve. It will then perhaps be found that the "breakdown" of accommodation is a feature common to all nerves and is by no means confined to mammalian nerves.

After the decompression, the capacity for accommodation tends to decline towards zero, and the nerve may entirely lose that capacity for some length of time, depending on the duration of the preceding ischemia. The capacity for accommodation thus at first tends to increase during ischemia, but is afterwards reduced below the original value. In the typical example shown,

the slope rose from 19 to 234 after ischemia for 19 minutes, but fell after the decompression down to about zero. A remarkable achievement on the part of the nerve!

Now, how can the gradual bending of the accommodation curve described above be accounted for? A possible explanation is that the index shifts. In that case the initial part of the curve, which is affected earliest and most markedly, and which corresponds to stimulation with the weakest current, would represent the coarsest motor fibres. The coarser fibres, as we know, have a low threshold and, as indicated in the introduction, are also most sensitive to ischemia. The more horizontal part of the curve, which is not affected so rapidly at first, might then be conceived to represent a finer group of fibres, with a higher threshold, poorer accommodation and greater resistance to ischemia. This may be the explanation of the striking change in the configuration of the curve.

3. Comparison between the changes in accommodation in long and short nerve fibres.

It might be expected that the greater sensitiveness of the long nerve fibres to ischemia, which manifests itself in the earlier onset of paralysis and in greater variations in threshold, would be noticeable also in the changes of accommodation. This is in fact the case. Relative changes in accommodation, under the same experimental conditions as in the determinations of threshold just reported, are shown in Fig. 12. As the accommodation curves taken during ischemia are bent, and not linear, in their initial part, the accommodation is evidently not well defined. In order to obtain at any rate a serviceable relative gauge, the accommodation was computed from the slope, drawn from the origin through the point marking the threshold value of a stimulating current with a time constant of 30 msec. This procedure seems permissible, as the two curves which we are comparing are bent. It should be borne in mind, however, that this is no absolute value, commensurable with the accommodation values obtained in straight curves. After the decompression, the curves seem to be rectilinear: but, in view of the rapid changes in rheobase and accommodation, the slope was determined separately in this case too, though only with a stimulating current with the rising time of 48 msec.

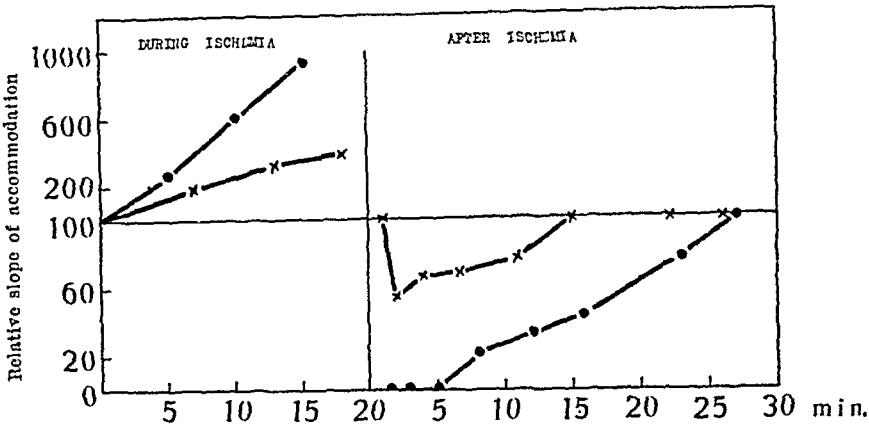


Fig. 12. Comparison between the changes of accommodation in long and short fibres of the ulnar nerve at the elbow during and after ischemia ●——●——● Relative value of accommodation in fibres to *musc. inteross. dors. 1.* ×——×——× Relative value of accommodation in fibres to *musc. flex. carp. uln.*

As shown by the Figure, the slope rises more rapidly for the long fibres, but after the decompression is maintained about zero for some minutes. As regards the shorter fibres, the variations —as was the case with the rheobase — are considerably less, and the zero value is not reached within such a comparatively short time as 20 minutes. After the lapse of 30 minutes, however, zero is usually reached in these fibres also. The changes are substantially similar, though they manifest themselves more rapidly in the longer fibres.

4. Comparison between the changes in accommodation distally and proximally in the same nerve fibres.

A stimulating electrode was placed at the elbow and another at the wrist. The changes in accommodation at these two points were then followed. The index adopted was a minimal twitch in *musc. interosseus dors.* I. (Similar results were obtained when a twitch in the other hand muscles supplied by the ulnar nerve was taken as an index.) The accommodation was found to be quite similar at these two points. Thus, as shown by the typical example in Fig. 13, the accommodation, measured similarly as in the preceding test, rises, on compression, from the same initial value with considerably greater rapidity proximally than distally.

When the blood-flow is released, the accommodation in this

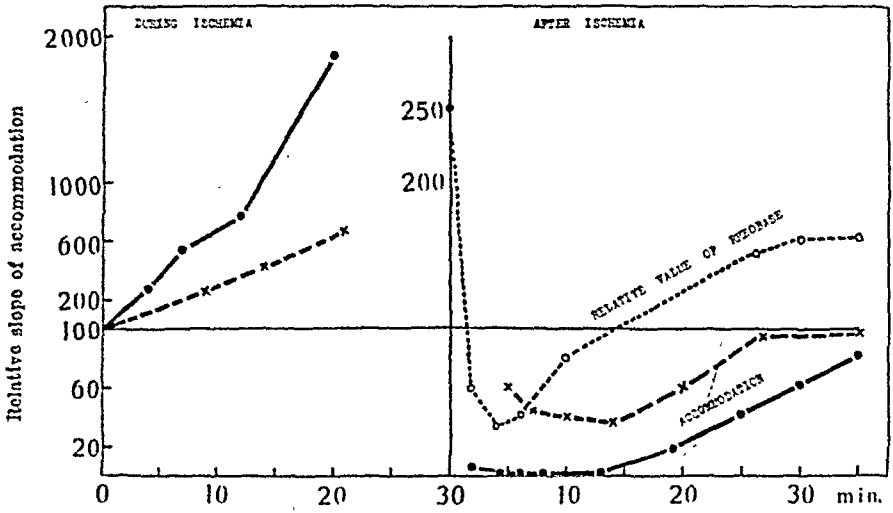


Fig. 13. Comparison between the changes of accommodation at the elbow and at the wrist in fibres to *musc. inteross. dors. I* during and after ischemia.
●——●——● Relative value of accommodation measured at the elbow.
× - - - × - - - × Relative value of accommodation measured at the wrist. Dotted line: relative value of rheobase at the elbow.

case falls proximally down to zero for about 10 minutes after ischemia with a duration of 30 minutes. Despite the lengthy ischemia, it does not reach zero distally. The accommodation may thus vary at different points of the same nerve fibres, because the anoxemia does not affect the fibres uniformly along their entire length, but in the main proximally, with a diminishing effect towards the periphery. Had the accommodation been measured proximally in a nerve and at a motor point, one might have been tempted to speak of a "hetero-accommodation" of pathological origin between muscle and nerve, in the phraseology of the complicated doctrine of chronaxie. This, however, would have been a mistake. The changes in accommodation were measured also at a motor point and they nowise differ from those in the adjacent stretch of nerve in cases where curves were obtained without breaks, which, however, is unusual. The occurrence of breaks in the curve likewise conduces to give the appearance of "hetero-accommodation", if we confine ourselves to determining merely a single point on the accommodation curve, and if the accommodation is computed from that point, just as the chronaxie is computed from a single point on the strength-duration curve.

It might be objected that the difference in accommodation prox-

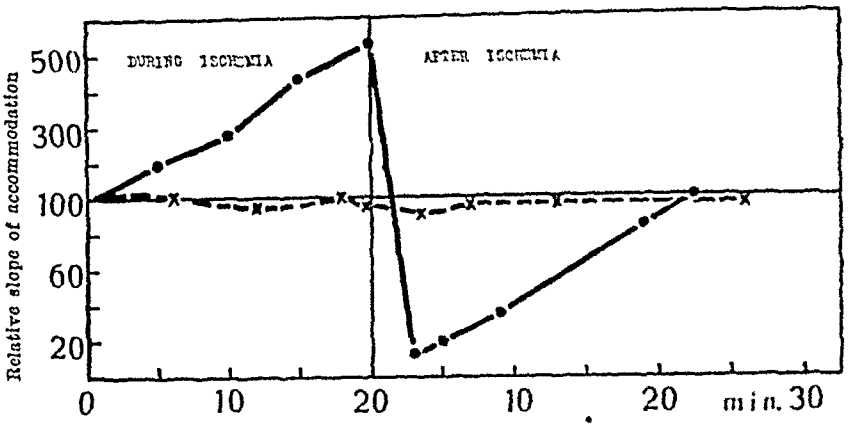


Fig. 14. Changes in accommodation proximally and distally to the cuff in fibres of the ulnar nerve to *musc. inteross. dors. 1.* ×——×——× proximally to the cuff. ●——●——● distally to the cuff.

imally and distally is merely apparent, and that the accommodation observed on the stimulation of a certain point in the nerve is not the accommodation precisely at that point, but that its value in this case too depends on the state of the nerve between that point and the muscle serving as an index, and possibly also on changes in the index itself. However, as shown by the following tests, this is not the case.

Fig. 14 shows how the excitability has been followed proximally and distally to a pneumatic cuff placed round the upper arm. The proximal part of the nerve is not exposed to ischemia, whereas on the greater part of the stretch between it and the muscle the accommodation varies within wide limits. As will be seen, this variation does not affect the accommodation proximally.

It may be regarded as certain that the changes in threshold are likewise most marked proximally; but it is more difficult in this case exactly to determine the difference, as the excitability is so different from the outset: the rheobase at the wrist is usually two or three times that at the elbow. It is, however, easy to show that a difference exists, seeing that, as found by Lewis and associates with a faradic current, the excitability during protracted ischemia ceases earlier proximally than distally.

In Fig. 13 the changes in the rheobase have been inserted for the purpose of comparison with the accommodation. Their general course is similar to that of the accommodation. Both rise during the compression and then temporarily fall below the initial value, but the succeeding rise is more rapid as regards the rheobase,

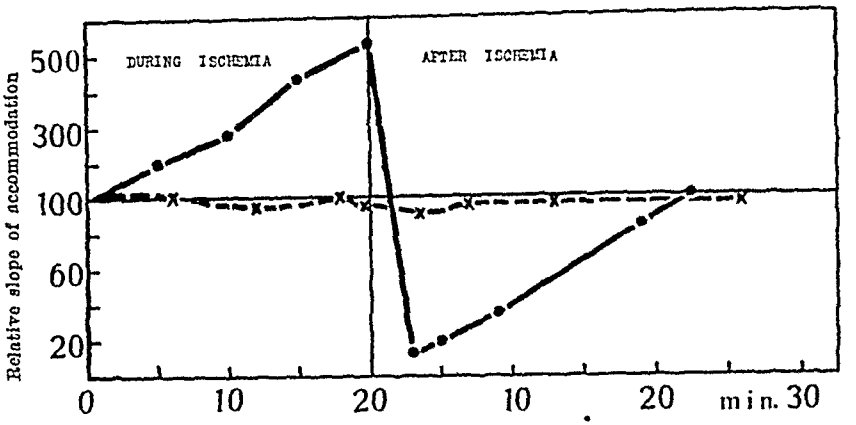
which thus to some extent changes, evidently irrespective of the accommodation. In the stage after the compression, when the accommodation and the rheobase are at their lowest, fibrillar twitches occur as well as the slow contraction mentioned by BOURGUIGNON and LAUGIER. This phenomenon will be discussed in the next chapter.

5. The nervous mechanism of the fibrillation-fasciculation.

As observed by REID (1931), fibrillar twitches occur after the decompression, especially in the muscles of the hand. Their number and distribution is subject to certain individual variations. In connection with the previously reported investigations, they were observed after ischemia for 15 minutes, produced by a pneumatic cuff placed and distended round the upper part of the arm of 41 apparently healthy subjects between the ages of twenty and thirty. In 8 cases nothing noteworthy could be observed. In 4 cases, on the other hand, contractions which were almost continuous, and which involved the entire muscular system in the thenar and hypothenar eminences and the *musc. interossei*, were distinctly noticed. Relatively slow, irregularly oscillating movements were observed on the surface of the affected muscles. At the same time the muscles were throughout moderately contracted, the fingers assuming the typical *"main d'accoucheur"*, which, however, could be mastered by a voluntary effort.

This extreme form of contraction is evidently identical with the "Trousseau sign" of the kind occasionally observed by PHLEBS (1913) and NOTHMAN (1937) after the compression, even if the genuine sign had not occurred during the actual compression. In cases where the contractions are intensive, they are also distributed over a larger area: they may spread even to the muscles of the forearm. In the other cases investigated, the contractions were intermediate in intensity between these two extremes.

As a rule a few slight twitches of short duration are observed in the fingers. Each twitch involves a bundle of muscle fibres of a few millimetres. The structure which contracts is thus considerably larger than a muscle fibre, which can scarcely be observed with the naked eye: it is, in fact, comparable in size with a muscle



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fascicle. As pointed out by DENNY-BROWN and PENNYBACKER (1938), in regard to similar motor phenomena, "fasciculation" is therefore a more apposite designation than the usual clinical term "fibrillation".

The frequency of the twitches moreover depends on the duration of the preceeding ischemia. *If it lasts less than 8 minutes, it will* scarcely give rise to any twitches at all. It should further be noted that compression high up on the upper arm is far more effective than it is further down.

In short, it may be stated that the contractions are analogous to the form of "tingling" which occurs after the compression. The latter, as previously mentioned, increases in intensity and distribution, the longer the duration of the ischemia and the higher up the pneumatic cuff is placed (LEWIS and associates, 1931, ZOTTERMAN, 1933).

There is also good reason to presume that both these phenomena are brought about by the same mechanism. Thus the fasciculations are not produced by direct stimulation of the actual muscle, as REID supposed, but arise much in the same way as "tingling", when the blood flows back to the proximal part of the ischemic nerve. This can be easily demonstrated if, before the decompression, a second pneumatic cuff is placed and distended distally to the original one, which is not removed till afterwards. It will then be found that, though the blood is thus prevented from reaching the periphery, these fasciculations nevertheless occur, as also their extreme variant, the Trousseau-like phenomenon. *Fasciculations are thus caused by impulses set up in the nerve itself, and particularly in its proximal part, during its recovery.*

As already indicated, *it is in the proximal part of the nerve that the response to electric stimuli shows the most marked and characteristic changes.* A few minutes after the decompression, the rheobase is lowered (i.e. the excitability is increased), whilst the capacity for accommodation approaches zero. Whether and when it actually reaches zero, as also the intensity of the contractions, on the one hand, and of the tingling, on the other, will depend on the duration of the ischemia. If the stimulating electrode is placed below the pneumatic cuff, in order to determine the excitability in the most proximal part of the nerve, it will be found that the fasciculations commence a few minutes after the decompression, when the capacity for accommodation is at or near zero. The twitches cease when the accommodation again rises.

From experiments on frogs nerve, it is known that, when the capacity for accommodation is at or near zero, whilst the rheobase is lowered, the motor nerve begins to discharge spontaneously (see the historical section I), fascicular twitches being at the same time observed.

Also in human motor nerves, spontaneous activity seems to show a similar behaviour, or rather, *the change in excitability which is quantitatively expressed by the accommodation curve gives rise to spontaneous discharges.* It is also more than probable that the excitability of the sensory nerve fibres changes in an analogous way. Spontaneous activity presumably occurs not only in the fibres which give rise to "tingling", but also in the fibres of the muscle proprioceptors. It might thus be contended that the fasciculations were nothing but a proprioceptive reflex. There is, however, no known evidence in support of the supposition that the muscle spindle apparatus could give rise to non-synchronized activity of such small muscular units as those of fascicular size. It may therefore be suggested that the fasciculations have their origin solely in the motor fibres.

The slow muscle contraction observed by BOURGUIGNON and LAUGIER (1923) on the stimulation of the nerve is likewise explained by the poor accommodation. The nerve cannot adapt itself rapidly enough to the stimulus, whence the response takes the form of iterative discharges, with a consequent slow contraction of the muscle, or a tetanus.

6. Some contributions to the nervous mechanism of two different forms of paresthesia which occur during and after arrest of the blood-flow.

A. *During ischemia.* About 2 minutes after the arrest of the blood-flow, a paresthesia normally develops in fingers and hand. As it seems to have been rather overlooked, it may be desirable to subject it here to closer analysis. It is subjectively identical with the sensation produced by stimulating, with a constant current, a purely sensory or mixed nerve, as described on p. 49. It thus has its origin in coarse fibres and is of a tactile character. That it is identical with the paresthesia induced by stimulation with a weak electric current, one can easily convince oneself by the following experiment: — During the course of the paresthesia

in the hand, stimulate e.g. the ulnar nerve with a current the strength of which is somewhat above the threshold for the breakdown of accommodation of the tactile fibres. Within the area of distribution of the nerve, no change in the *character* of the paresthesia can then be observed. The only change that occurs is an increase in intensity, which, on close analysis, appears to consist in an increase in the frequency of the vibrations and in the number of the little diffuse pricks which constitute the paresthesia. If this tactile paresthesia is of great intensity, it is locally felt as a "numbness" of the skin, as the experimental subjects often spontaneously remark. The appreciation of touch with a piece of cotton-wool is evidently diminished. Whilst the perception of light touch is thus reduced, the tactile stimulus, on the other hand,

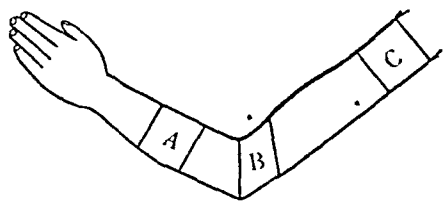

Fig. 15. Sketch illustrating the placing of the pneumatic cuffs. See the text.

reduces the intensity of the paresthesia. If, for example, a piece of cotton-wool is passed over the hand, the paresthesia will be momentarily abated, but immediately afterwards may perhaps be felt somewhat more intensely than before.

Tactile paresthesia begins at the periphery and spreads centripetally, afterwards vanishing reversely to the order of involvement. Minor deviations from the centripetal spread are rather common. As a rule it begins in the finger-tips, but it may also start in the thenar or hypothenar eminence. Sometimes it spreads most rapidly on the palmar side of the hand, sometimes on the dorsal side. Its intensity, however, is almost always greatest on the palmar side, which may be due to the fact that the touch sense is best developed there. The upper limit of the tactile paresthesia is as a rule approximately at the wrist. It begins, as already mentioned, about 2 minutes after the compression and vanishes after the further lapse of 4—8 minutes.

In order to determine in what part of the nerve the tactile paresthesia originates, the following experiment was made. The arm was immersed in water heated to 37° C. The hand, however, was held above the surface of the water, as the tactile paresthesia

is then more easily perceived. Pneumatic cuffs were then placed and distended at three different places on the arm. A clear description of the results of the experiment is given in the following protocol:

Time.

0′. Cuff A. is distended up to 300 mm Hg round the middle of the forearm.

2′. Scarcely noticeable tactile paresthesia at the end of the thumb.

5′. The tactile paresthesia had cleared away. Another cuff B. was distended just proximally of the elbow to 200 mm.

6.5′. Tactile paresthesia of moderate intensity spreading over the volar side of fingers 1, 2 and 3.

10.5′. The tactile paresthesia had cleared away. Another cuff C. was distended to 200 mm as high up on the upper arm as possible.

12′. Tactile paresthesia of considerably greater intensity than the preceding commences in the fingers, spreads over *vola manus* and, with somewhat lesser intensity, over the back of the hand up towards the wrist.

19′. The tactile paresthesia had cleared away. Cuffs B. and C. were removed. No redness of the skin distally to A.; anesthesia in the finger-tips gradually developing and spreading centripetally.

In the different places on the upper arm, nerves and blood-vessels can be compressed with equal effectiveness: it is therefore permissible to compare the compression effects at those points. On the forearm, on the other hand, they are less accessible, being protected by the bones. It should be noted, however, that the effect of the compression does not increase if the pressure is raised beyond the limit required in order to produce an ischemia in the nerve. As shown by Lewis and associates (1931) it is the ischemia, and not the pressure as such, that is effective. Since no redness of the skin was observed distally to cuff A., when C. and B. were removed (see the protocol) and an anesthesia had developed, it may be presumed that the blood-flow had been effectively arrested also during compression under the cuff A. The results of ischemia produced on compression of the forearm are therefore comparable with those obtained on the upper arm.

The experiment thus shows that tactile paresthesia spreads further and acquires greater intensity the further proximally the compression is made; this, in spite of the fact that the greater part of the stretch of nerve situated peripherally thereto cannot give rise to any paresthesia, having been already subjected to ischemia. Tactile paresthesia must therefore originate mainly in the most

proximal segment of the nerve subjected to ischemia. On compression of the upper arm, however, it is evidently produced to some extent also on the stretch of nerve passing down to the middle of the forearm.

As might have been expected, its place of origin is thus mainly in the most proximal parts of the longest tactile fibres, which, as we know, are most rapidly paralyzed. At these places on the nerve a spontaneous activity arises, which is probably identical with the spontaneous firing in the A fibres of cat nerve asphyxiated *in vitro* (as described by LEHMANN, 1937 b). In the peroneal type of cat nerve, it occurs during the first 8 minutes in connection with a lowered rheobase. In the saphenous nerve on the other hand, no spontaneous activity is observed.

In man, the proximal part of the nerve fibres in the arm which run furthest out to the periphery would thus correspond, as regards their reaction, to the peroneal type, the other fibres to the saphenous type.

In the leg, the reaction to ischemia is similar to that previously noted. Also in man, as shown by THOMPSON and KIMBALL (1936), the rheobase for sensory fibres is lowered during an ischemia lasting 10 to 15 minutes. It might further be expected that the accommodation would be poor or absent, as in the spontaneous activity of frog nerve (see p. 16). However, it was not found possible to measure the accommodation in such cases, as the thresholds could not be determined. The figures obtained are unduly high, seeing that the electric stimulus must override the already existing activity in order to be perceived at all.

The tactile paresthesia above described, including the numbness, occurs in migraine, as the author has had occasion to observe on himself. Just as in the case of arrested blood-flow to the arm, it usually begins, as we know, at the periphery of the limbs and spreads centripetally. It occurs also on the lips, face and tongue. It is presumably produced, as is generally supposed, by ischemia of the sensory pathway or cortex, due to arterial spasm.

The fact that the fibres running to the most peripheral parts are the most sensitive to ischemia thus probably holds good right up to the cortex. This is indicated by the fact that the hand and foot suffer most in vascular lesions, such as hemiplegia due to cerebral hemorrhage, etc.

Tactile paresthesia is also responsible for the "tingling" and

numbness which are ordinary symptoms in tetany, and which will be described in the following chapter.

B. *After ischemia.* Now, in what relation does the above described tactile paresthesia (No. I) stand to the form of tingling (No. II) which occurs *after* the decompression? Subjectively, the symptoms are quite different, as one can easily verify on oneself by arresting the blood-flow with a pneumatic cuff for about 15 minutes and comparing results. This, *en passant*, is a suitable method for clinical use, if it is desired to ascertain from what form of paresthesia a patient is suffering. As a rule it is rather difficult from his description of the symptoms to get any clear idea of its nature. It is easier for the patient to indicate whether the paresthesia resembles the one or the other of the types which occur during ischemia, or whether it is possibly of quite a novel character.

If now paresthesia No. I was a tactile paresthesia, it is rather difficult to believe, as supposed by LEWIS and co-workers (1931), that this was the case also with No. II. It is indeed probable that changes in the "impulse pattern" set up for the same kinds of nerve fibres may appreciably modify the subjective sensation. This perhaps explains why the tactile paresthesia is not distinctly felt as a sensation of touch. The difference between No. I and II, however, seems to be too marked to be explained in this manner. The supposition of ZOTTERMAN (1933) that in No. II we are not concerned with the tactile fibres, but with other fibres of finer calibre, conducting pain, is thus borne out.

It should further be observed that "tingling" II is not a uniform paresthesia: one can distinguish at least two components. The main component consists of innumerable, well-localized short pricks, which are of a sharp and distinct character, not soft and diffuse as in tactile paresthesia. Nor are they synchronized into a vibratory sensation. The other component is an irradiating stinging sensation of a distinctly painful character and of considerably longer duration.

If we now attempt to discuss the nature of these components, it may be stated that the second component subjectively well corresponds with the sensation of the delayed pain of a needle prick, which, as already shown, is conducted by the fine slow-conducting fibres of the C class (see the Introduction p. 53).

If an endeavour is made to reproduce "tingling" II by the stimulation of a cutaneous nerve with a constant current, we shall find that tactile paresthesia can be produced by currents of low strength. If the strength of the stimulating current is then raised to 5—10 times the rheobase for the tactile fibres, the paresthesia will take the form of a sensation corresponding rather well to the main component in "tingling" II. The stinging sensation, however, cannot be reproduced, even if the current strength is raised to 20 times the rheobase for the tactile fibres. Strong currents would cause considerable pain under the stimulating electrode, which would prevent an analysis of the sensation in the area of distribution of the nerve. That the stinging sensation cannot be produced under such conditions in response to an electric stimulus is not at all surprising. It would in fact have a very high threshold, if, as assumed, it is identical with the delayed pain conducted by the C fibres.

It may also be asked whether the pricking sensation produced with a current strength of 5—10 times the rheobase for the tactile fibres is really a new sensation obscuring the preceding tactile paresthesia, or whether it is merely a tactile paresthesia of higher intensity.

The first-mentioned alternative is borne out by an investigation of HEINBECKER, BISHOP and O'LEARY (1933). Human sensory nerves were directly stimulated with a needle as the cathode. Condenser changes of a frequency up to 60 per second were used as stimuli. At a threshold of 5 to 6 times the threshold for the tactile sensation, a painful sensation, which obscured the first-mentioned one, was produced. Also on the stimulation of dog nerve a distinctly painful sensation was produced by currents of the same relative strength. On the electroneurogram of excised human nerves and in exposed dog nerves, the potentials corresponding to the two sensations could then be identified. The tactile sensation was thus conveyed by the large and fast fibres, the painful one by a potential component with a slower conduction rate, from 30 to 15 metres per second.

There is thus some reason to presume that "tingling" II is a pain paresthesia, combined with another component which has its origin in fibres with a conduction speed of 30 to 15 metres per second.

The change in the electric excitability may be presumed to be the same as in the motor fibres, notably a lowered rheobase and an

accommodation about zero, giving rise to spontaneous activity in the motor fibres of human nerves and frog nerve.

7. Summary and Conclusions, Section IV.

Ischemia caused by compression affects the motor fibres so that the rheobase diminishes during the first 10—20 min. and then rises above the original value, as a rule first slowly, then with increasing rapidity. Complete lack of response to the stimulating current is scarcely reached before 30 min. As soon as the blood-flow is released, the rheobase is again rapidly lowered: after a lengthy ischemia of 30 min., down to about 50—70 per cent. below the original value. Then follows a second rise of the rheobase above its initial value. On this level it is maintained for some time, the extent of which depends upon the duration of the preceding ischemia.

In general, the accommodation changes parallel with the rheobase. Thus it rises during the ischemia, in order afterwards to fall below the original value. After a sufficiently long period of ischemia, accommodation drops to about zero, parallel with the similar change in the rheobase. Afterwards, however, accommodation rises in a lesser degree than the rheobase and does, not as the latter, surpass its original value.

Ischemia affects the slope of the accommodation curve so that it tends to become steeper. This tendency is most pronounced in the initial part of the curve and affects its latter portion to a lesser extent, so that the accommodation curve as a whole deviates from its initial rectilinear part and bends in a gradually diminishing slope. In tests on man this type of curve must be regarded as pathological. It has never been observed under normal conditions.

After an ischemia of 20—30 min., steep accommodation curves are obtained of a type which in excised frog nerve is held by many authors to be normal. Under such conditions there is neither breakdown of accommodation nor a cathodal closing tetanus in man. The nerve therefore requires a minimal current gradient for excitation. As the existence of a minimal current gradient in man is a pathological phenomenon, caused by circulatory disturbance, we must ask whether a similar explanation does not hold good also of excised animal nerve. Breakdown of accom-

modation, regularly seen in man and other mammals, is probably a feature common to all nerves.

After decompression, accommodation tends to decline to zero, and the nerve may entirely loose its capacity of accommodation for some length of time, depending on the duration of the preceding ischemia.

Both during and after ischemia the changes in rheobase and accommodation are best marked in the proximal part of the longest fibres of the ischemic nerve. Thus accommodation may be different in different parts of a nerve under pathological conditions. Possibly some difference exists even in normal nerve along its course, though this could not be shown by the method of investigation.

Some minutes after the release of the blood-flow, fascicular contractions appear in the muscles of the hand. It has been shown that they are caused by spontaneous activity generated in the highly sensitive proximal portion of the ischemic nerve. These fasciculations coincide with the minimal values for accommodation and rheobase, which likewise reach their lowest point in this same region of the nerve. This type of spontaneous activity in motor nerves of man is thus characterized by a low rheobase and low or absent accommodation. The low accommodation also explains the slow muscle contractions already obtained by stimulation of the nerve at rheobase strength, a phenomenon first observed by BOURGUIGNON and LAUGIER.

The fasciculations may be regarded as the motor equivalent to the paresthesias which originate in the same proximal portion of the nerve after ischemia. Consequently, the sensory phenomena termed paresthesias, are due to spontaneous activity caused by a lowering of the rheobase and diminution of the accommodation, such as was found in motor nerves.

Detailed analysis of these paresthesias has given the following results: 2—7 min. after the arrest of the blood-flow, a touch paresthesia originating in the fibres for light touch is observed. During the recovery after decompression, a paresthesia consisting of two components is perceived. The one component may be localized in finer fibres than those transmitting light touch, though the character of the sensation cannot be determined with certainty. The other component is definitely in the nature of a sensation of pain.

V. Nerve accommodation in tetany. The influence of the reduction of ionized calcium on the nerve's reaction to ischemia; the nervous mechanism of typical paresthesia; spasm and fasciculations in tetany; the mechanism of the Trousseau sign.

1. The accommodation in some clinical cases with symptoms of tetany.

In two clinical cases of tetany, SOLANDT (see the historical section, p. 18) observed a poorer accommodation, measured on the ulnar nerve, than normal. As shown by some clinical cases which will now be reported, this observation has been on the whole confirmed by the author.

Case 1. Maria P., aged 31. Parathyreoprival tetany. Fell ill in 1939, in connection with thyroidectomy. Since then she had almost daily (especially in the morning) been subject to stiffness and tension in her fingers. Especially during menstruation, or in infections accompanied by fever, the feeling of tension usually increased, typical tetany spasms occurring in the fingers. Neither spasm nor the feeling of tension had occurred in other muscles than those of the hand. She complained of a frequently occurring feeling of numbness in her hands and forearms, but had not noticed any actual paresthesia. Her vision showed nothing noteworthy.

She was examined by the author on the 15th May 1942. A few hours before this examination she had had typical spasms in the fingers. The feeling of tension and some stiffness still persisted. In the thenar and hypothenar eminences and in *musc. interossei* moderate fasciculations were observed. Chvostek sign positive bilaterally in all three branches of the facial nerve. Trousseau sign positive. Blood calcium 8.5 mg %.

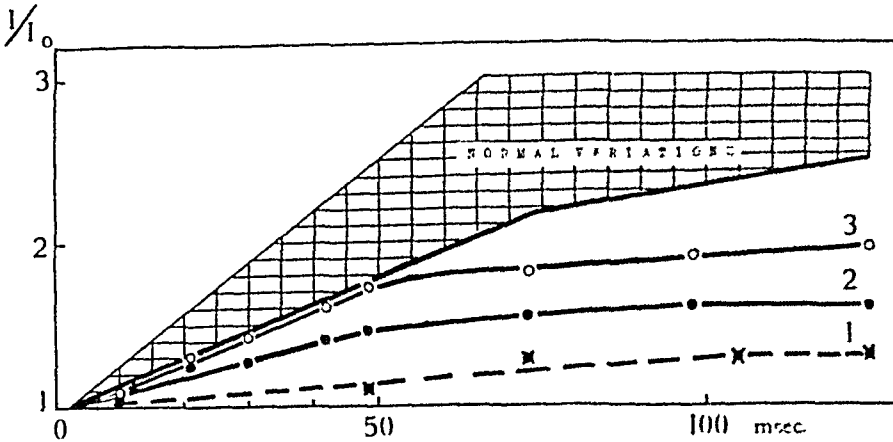

Fig. 16. Accommodation curves recorded from the tetany cases 1, 2 and 3 on the ulnar nerve excited at the elbow. Curve 1, index: minimal twitch observed at *musc. inteross. dors. 1.* Rheobase 0.46 mA. Accommodation slope: 3. Curve 2, index: minimal palpable twitch in the tendon of *musc. flex. carp. uln.* Rheobase 0.5 mA. Acc. slope 10.5. Curve 3, index as in curve 2. Rheobase 1.5 mA. Acc. slope: 15.

At the time of examination the patient was evidently on the verge of manifest spasms in the small muscles of the hand. (This was the only clinical case which I have had occasion to examine during the actual course of muscular irritation.) On stimulation of the ulnar nerve at rheobasic strength at the elbow, a slow tetanic contraction in *musc. interossei dorsales, adductor pollicis* and *abductor digiti* 5 was observed. The rheobase was 0.46 mA and the initial accommodation slope, measured at *musc. inteross. dors.* 1 and *musc. digiti* 5, which were most easy to observe, was merely 3. If an observed twitch in *musc. flexor carpi ulnaris* was, instead, taken as an index, the contraction was found to be normal. The rheobase was then 0.5 mA, the slope 14 and the breakdown of accommodation occurred at 2.5 rheobases. The radial nerve, with a twitch in the tendon of *musc. extensor pollic. longus* as an index, had a rheobase of 1.7 mA and a slope 18. It was also interesting to observe that, if the ulnar nerve was stimulated at the wrist, the response in the *musc. interosseus* to the rheobasic current was precise and rapid, and that the accommodation measured at this point was better than at the elbow.

On the following day the feeling of tension and the fasciculations had vanished, but the accommodation in the nerve fibres of *musc. inteross. dors.* was still bad, namely 8, as compared with 14 in the fibres of *musc. flexor carpi ulnaris.* In the peroneal nerve the corresponding figure was 14, in the facial nerve 20.

Summing up, it may thus be stated that, when muscular irritation occurred in the form of fasciculations and stiffness in the small muscles of the hand, the capacity for accommodation, as measured at the elbow in the nerve fibres of these muscles, was almost absent. Further distally it was somewhat better. In other examined nerves, the accommodation was less affected.

Case 2. Ellen L., aged 37. Cancer pancreatis. She fell ill on the 21th Dec. 1942 with dull pains in the epigastrium and vomiting. The vomitings continued, and after the 22nd January 1943 had a daily volume of about 2 litres. During this period the patient was now and then subject to paresthesia and a feeling of numbness in her face, hands and legs. Blood calcium: on the 7th January 9.5 mg %. On the 26th January the chloride of sodium in the serum was 295 mg % and the chlorine 180 mg % (normal values 560—630 mg % and 340—380 mg %, respectively). Chvostek and Trousseau signs positive throughout. X-ray examination of the stomach revealed that it was filled with fluid but not dilated and otherwise showed no pathological changes. The patient steadily declined in health and died on the 17th February. The postmortem showed a cancer of the pancreas which had constricted the intestine.

This is a typical case of gastric tetany, resulting from prolonged vomiting, with the loss of acid valencies and consequent alkalosis (see NOTHMAN, 1937). The accommodation was determined on the ulnar nerve, with a palpable twitch in the tendon of *musc. flexor carpi ulnaris* as an index. On the 25th January it was 10.5, with the breakdown at 1.8. The rheobase was 0.5 mA. On the 30th January the corresponding values were 10, 1.6 and 0.5. At the examinations the Chvostek and Trousseau signs were positive, but there was no manifest tetany. The patient declared that her paresthesia corresponded with the paresthesia and feeling of numbness which occurred about a minute after the blood-flow had been arrested with a pneumatic cuff round the upper arm. The paresthesia from which she had been suffering was thus a tactile paresthesia.

Case 3. Viola O., aged 35. Parathyreoprival tetany. In connection with thyroidectomy in 1933, she had suffered from typical spasms in her hands, which continued for a week or so. Since then no manifest symptoms of tetany, no spasms or paresthesia. Since 1939 increasing diminution of vision, which was ascertained to be due to a cataract in both eyes, for which she was operated in 1941. Blood calcium: about 7 mg % during several years, on the 7th January 1943 6.8 mg %.

At the medical examination on the 6th February 1943 positive Chvostek and Trousseau signs. The initial accommodation

slope, measured on the ulnar nerve, with a palpable twitch in the tendon of *musc. flexor carpi ulnaris* as an index, was 15. The breakdown of accommodation occurred at 1.8 times the rheobasic strength, the rheobase being 1.4 mA.

Case 4. Elsa E., aged 56. *Status post ectomiam adenomatis parathyreoideae.* Operated on the 7th December 1943, when a parathyroid adenoma, of the size of a brown bean, was extirpated. On the day after the operation, paresthesia and numbness in the hands, lower legs and face. Afterwards also occasional spasms in the hands and round the mouth. Blood calcium: on the 12th December 7.5 mg %, on the 16th December 8.8 mg %.

At the examination on the 15th December 1943, positive Chvostek and Trousseau signs, but no manifest tetany. The patient declared that the paresthesia from which she had been suffering was of the same nature as that produced by the pneumatic cuff test. The initial accommodation slope on the ulnar nerve, with a palpable twitch in the tendon of *musc. flexor carpi ulnaris* as an index, was 15. The breakdown of accommodation occurred at 2.8, the rheobase being 0.7 mA.

Case 5. Maja W., aged 37. Parathyreoprival tetany. Fell ill in connection with thyroidectomy in 1931. During the first few years after the operation, daily spasms in the hands, a feeling of tension in the face as well as paresthesia in the face, arms and legs. Since 1936 had taken 1—2 cc AT 10 a week, with a noticeable improvement in her symptoms. Latterly she had suffered only quite occasionally from spasms in the hands and tension in the face, and somewhat oftener from paresthesia with typical localization. In connection with the paresthesia she had noticed a feeling of numbness. Blood calcium: on the 21st January 1944, 7.1 mg %.

At the examination on the 21st January 1944 the patient showed positive Chvostek and Trousseau signs. After the pneumatic cuff test, the patient declared that the paresthesia from which she had been suffering was of the same nature as the tactile paresthesia just experienced. The initial accommodation slope, measured on the ulnar nerve with a palpable twitch in the tendon of *musc. flexor carpi ulnaris* as an index, was 18.5. The breakdown occurred at about 3, the rheobase being 1.5 mA.

Summing up, it may thus be stated that in four out of the five cases of tetany, the figure for the initial accommodation slope is lower than the minimum figure, 16, measured in 100 normal cases. In the fifth case it was 18.5. The figure for the breakdown of accommodation, which is almost identical with the cathodal

closing tetanus, varies, broadly speaking, parallel with the figure for the initial slope (see Fig. 16). Thus the less steep the slope, the lower the value for the breakdown of accommodation.

In case No. 4, however, where the value for the initial slope was pathologically low, the breakdown occurred at about 2.8, a figure which lies within the normal variations 2.5—5. But this may be explained by the difficulty in determining it accurately, since threshold values with slowly rising currents are not easily computed, and the slope in this case bordered on the normal.

It may be questioned whether the determinations of accommodation have any practical diagnostic value when there is a reduction of ionized calcium. In order to settle this question, comparisons must be made with the other diagnostic methods employed in practice. The most commonly adopted electric method is to determine the thresholds to cathode and anode, which ERB (1874) showed to be lowered. ERB also pointed out that the figure for the cathodal closing tetanus fell, both absolutely and relatively to the cathodal closing twitch, as the difference between them diminished. Now, as above indicated, the cathodal closing tetanus is in reality nearly identical with the breakdown (see p. 30). If its value, as also that of the breakdown, is expressed in multiples of the rheobase, in order to eliminate the effect of the thickness of the subcutaneous fat, etc., — which so seriously reduces the importance of the absolute value of the thresholds as a diagnostic —, the cathodal closing tetanus is undoubtedly of the same value as the breakdown. However, both are difficult to observe and require, for exact determination, electro-myographic recording of the muscle action potential as an index, direct observation of the slow muscle contraction being unreliable.

The normal value of the rheobase on the ulnar nerve is stated by NOTHMAN (1937) to lie between 0.6 and 1.8 mA, which accords well with the values obtained concurrently with the accommodation in the normal cases reported on p. 31, if male as well as female subjects are included. The rehobase would then be pathologically low only in case 2. It is therefore, as is generally held, of small diagnostic value.

As regards the cathodal opening contraction, which after the investigations of MANN (1900) and THIEMICH (1900) has been used especially in examination of children, where it has proved to be of value, it can be stated that in none of my cases could it be observed to fall below 5 mA, which is usually regarded as the normal

lower limit. It is quite possible that it was actually much lower, but, owing to the marked cathodal closing tetanus, the muscle was contracted, so that no opening twitch could be observed.

TEZNER (1924) recommends that, in such cases, the current should be maintained pending the cessation of the closing tetanus, whereupon it would be possible to observe the opening contraction alone. This procedure, however, would involve the introduction of a source of error arising from the additional electrotonic changes which take place during the action of the current, and which are dependent on its strength and duration. It is quite easy even in normal subjects to produce an opening contraction under 5 mA, provided that a current of the requisite strength is allowed to act for a sufficient length of time. This artificial interference is therefore open to serious objection.

Thus in all these cases the cathodal opening contraction was valueless as a diagnostic. This is in striking contrast to what is observed in children, where it is often lower than the closing tetanus, which therefore does not usually impede the determination. This surprising distinction is presumably due to some difference between the peripheral nerves of children and adults.

In judging the comparative value fo the Chvostek and Trousseau signs as a diagnostic, it should first be noted that both were positive in all five cases. Now, as is generally known, the Chvostek sign is positive in a rather high percentage of adult normal cases. I therefore have the same inpression as that of certain other investigators (e.g. FALTA, 1928), that, unless the symptom is very pronounced, with a marked contraction in all the branches of the facial nerve, its diagnostic value is very limited.

As regards the Trousseau sign, on the other hand, no data are available as to how frequently, if indeed at all, it may occur among what we usually call normal cases. On examination of 40 medical students and hospital nurses, an indication of it was observed merely in a single case, whereas more or less marked Chvostek signs were seen in 10 cases. In 8 additional cases the facialis phenomenon was also observed. (The Chvostek sign is defined here as a twitch elicited from one of the branches of the facial nerve by tapping that nerve anterior to the auditory meatus, and the facialis phenomenon as a twitch produced on tapping the region between the zygomatic arch and the corner of the mouth.) See NOTHMAN, 1937.

Thus, so far as can be judged from the relatively small number

of cases reported above, the Trousseau sign would seem to be the most reliable of these diagnostics. Compared with the various electric methods of determination, it has the additional merit of being much simpler. The performance of this test requires neither special apparatus nor special training. It should be noted here, however, that, as will be shown further on, the test is most effective if the pneumatic cuff is placed as high up as possible on the upper arm.

It may be desirable to point out that the above discussion refers to conditions in adults.

2. Nerve accommodation in tetany produced by forced respiration.

As first shown by GRANT and GOLDMAN (1920), all the symptoms of tetany can be produced, in a convenient way, by hyperventilation. In order to study the excitability in the nerve during or near the stage where its muscle shows symptoms of irritation, the accommodation and the rheobase were studied on the ulnar nerve of 5 experimental subjects during forced respiration. Exploring electrodes were fixed firstly at the axilla and secondly on the wrist, in order to test the changes which take place in the nerve proximally and distally. In both cases a minimal observed twitch, located at the same place in *musc. interosseus dorsalis* 1, was taken as an index of the excitatory effect. It may therefore be presumed that the excitability was investigated on identical nerve fibres.

In these tests no certain difference in accommodation proximally and distally could be observed at the outset. As the experimental subject cannot keep the arms quite still whilst taking as deep breaths as possible, the rheobase values are somewhat uncertain. In these circumstances the electrodes are liable to shift their position, which, as already shown (p. 27), affects the rheobase, but does not appreciably modify the figure for the accommodation.

Hyperventilation for a few minutes, as is well known, produces a feeling of tingling, which begins in the face and in the distal parts of the extremities, from which it spreads centripetally.

After a varying space of time (in my cases 5—20 minutes), partly depending on how far the experimental subject is able to

breathe deeply throughout, symptoms of muscular irritability begin to manifest themselves. First as a rule in the facialis region round the mouth, afterwards in the hands and lower legs. The first symptom is a feeling of tension, as though the muscle were striving to contract. It is interesting, however, to note that, despite a rather strong impression that the fingers are trying to assume the typical spastic position, there are at first no distinct objective symptoms of spasm. Somewhat later this feeling of tension passes into a manifest spasm. *The non-correspondence*

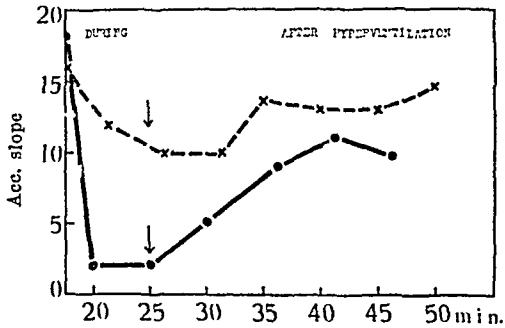

Fig. 17. Comparison between the changes in accommodation at the axilla and at the wrist in fibres of the ulnar nerve to *musc. inteross. dors.* 1. during hyperventilation. The dot-and-dash line shows the changes at the axilla, the broken line the changes at the wrist. The arrow indicates where the hyperventilation ceases.

between the feeling of tension and the spasm itself, makes it probable that the tension is really a paresthesia, i.e. started by inadequate stimulation and not caused by the spasm. In connection with the spasm, or perhaps most often when it has subsided, fascicular twitches were observed in the hand.

The measurements of accommodation were made distally and proximally on the nerve, especially at the stage when the muscle is on the verge of spasm. During the actual spasm the accommodation cannot be determined, seeing that, when a new contraction is superadded to that already existing, the thresholds are liable to be computed unduly high. This source of error makes itself felt especially during excitation with a slowly rising current.

The results of the determinations made proximally and distally during the feeling of tension in the hand are summarized in Table XVI. In cases 1 and 2, fascicular twitches were also observed. As the values change rather rapidly during deep respiration, the determinations were first made distally at the wrist and imme-

diately afterwards at the axilla, whereupon, for the purpose of control, a renewed determination was made at the wrist.

As will be seen from the table, the changes in the rheobase as well as in the accommodation are more marked proximally than distally. This is shown also by Fig. 17.

Table XVI.

The electrical excitability at the axilla and at the wrist on the motor nerve fibres of musc inteross. dors. 1 during forced respiration, when a sensation of tension is felt in the hand muscles.

Case		Before forced respiration		During forced respiration		
		Axilla	Wrist	Wrist	Axilla	Wrist
1.	Rheobase	1.6	1.6	1.1	0.2	1.3
A—n.	Acc. slope	18	16	12	2	10
	»Breakdown»	3	3	2	1.2	
2.	Rheobase	1.8	2.5	1.8	0.5	1.8
D—n.	Acc. slope	20	20	14	3.5	14
	»Breakdown»	3	3	3	1.5	3
3.	Rheobase	2.2	1.6	1.5	0.8	1.3
J—n.	Acc. slope	20	20	20	6	20
	»Breakdown»	3.4	4	3.3	1.8	3.7
4.	Rheobase	2.4	2.4	1.8	0.8	1.8
E—n.	Acc. slope	20	24	22	4	20
	»Breakdown»	3	3.8	2.8	1.4	3
5.	Rheobase	2.3	2.4	2.0	1.2	1.6
K—n.	Acc. slope	19	19	16	7	13
	»Breakdown»	3	3	3	1.9	3

In regard to the rapidity of the muscle contraction proximally and distally, a difference could at once be observed. The response to stimulation proximally was slow and tetanic even at currents of or about the rheobasic strength, the nerve being unable to set up any counteracting process. To stimulation distally, on the other hand, the response was rapid.[1]

[1] Apart from a single case, where it was slower than normal, though not in such a marked degree as proximally.

In spite of these quantitative differences, the changes were of similar character proximally and distally, reaching their maximum on the verge of the spasm, which is marked by a substantial fall in the rheobase, concurrently with a slowing-down of the accommodation. Proximally, especially in the cases with fasciculation, the accommodation was almost non-existent.

Attempts were also made to ascertain whether there was any difference in accommodation between the long and the short fibres, by stimulating the nerve at the elbow and taking alternative readings with a twitch in *musc. interossei* of the hand and in *musc. flexor carpi ulnaris* as an index. They showed that the slowing-down of the accommodation was probably more marked in the fibres of the hand. The difference, however, seemed to be less than that observed proximally and distally in the same nerve fibres. But, as the experimental subjects found difficulty in keeping their arms perfectly still while respiration was proceeding, the difference could not be determined with certainty.

All the experimental subjects, as also the author in tests on himself, noted that the typical tetany paresthesia, which begins in the face, hands, feet and lower legs, after a few minutes forced respiration becomes identical with the tactile paresthesia produced by stimulation with a weak electric current, or occurring shortly after the arrest of the circulation to a limb.

To sum up, during hyperventilation both rheobase and accommodation are lowered in the ulnar nerve. This change reaches its maximum when fasciculation — and, in a somewhat lesser degree, also when the feeling of tension — appears in the muscles of the hand. The diminished accommodation and rheobase or, what amounts to the same, the increased excitability is greater when measured at the axilla then at the wrist. The paresthesia felt is of a tactile nature.

3. The effect of a deficiency of ionized calcium on the nerve response to ischemia.

As shown in the preceding chapter, compression of the upper arm produces a paresthesia in the hand, due to spontaneous activity in the most proximal part of the longest tactile fibres of the ischemic nerve. After the ischemia, provided that it is of sufficiently long duration, a "mixed" pain paresthesia ensues, as well

as fascicular twitches, localized especially in the hand. Both these phenomena have their origin in the most *proximal part* of the nerve. Ischemia thus normally gives rise to symptoms of excitation.

In cases of deficiency of ionized calcium, as we know, another such phenomenon, namely the Trousseau sign, which takes the form of a tonic spasm, especially in the muscles of the hand, is also observed. Moreover — though this does not seem to be known —, certain changes occur in the reaction of the sensory nerve fibres. If a pneumatic cuff is placed and distended round the upper arm above the systolic pressure it will be found, after hyperventilation, for some minutes, that the tactile paresthesia begins earlier and has greater extension and intensity. This happens even before the Trousseau sign is observed. In all the above-reported cases of tetany the tactile paresthesia spread centripetally up to or above the elbow, whereas normally, as ascertained in 50 normal cases, it spreads only to about the wrist. *A deficiency of ionized calcium thus reinforces the effect of the ischemia*, the result being that the shorter tactile fibres, as well as the longer, begin to discharge spontaneously.

These findings accord well with those of LEHMANN (1937 b). He found that cat nerves of the saphenous type *in vitro*, which had shown no tendency to spontaneous activity during the first few minutes of asphyxiation, acquired such a tendency if they were made alkaline or *deprived of calcium*. Conversely, nerves of the peroneal type, which showed marked spontaneous activity during asphyxia, could be made to resemble the saphenous type of nerve by acidosis or by the administration of calcium. From this it might be concluded that the tactile paresthesia induced by ischemia would vanish, or at any rate subside, on acidosis or hypercalcemia. I have not, however, had an opportunity to ascertain whether this is actually the case.

The symptoms of irritability *after* ischemia are likewise considerably heightened. In all five cases of tetany the fasciculations after ischemia for 15 minutes were so marked that the hand assumed the typical spasm of the Trousseau phenomenon. This sign may therefore be of some diagnostic value. The tingling also shows considerably greater spread and intensity. Normally, after ischemia for 15 minutes it is located in the hand only. In all the cases of tetany, however, it spread to the whole forearm and upper arm up to the edge of the pneumatic cuff.

In addition to the intensification of the normally occurring

symptoms of irritability and the appearance of the Trousseau phenomenon, the nerve's increased sensitiveness to ischemia manifests itself also in the more rapid paralysis of the nerve. LEWIS and co-workers (1931) state that, if the arm is rendered ischemic by a pneumatic cuff round its upper part, the muscles of the hand are paralyzed in about 25 minutes; and that the sense of touch in the tips of the fingers begins to be paralyzed after 15 minutes.

These times accord well with my own experience. Among 15 healthy subjects subjected to ischemia for 25 to 30 minutes, the small muscles of the hand were paralyzed in 2 cases after 18 minutes, in the other cases after the lapse of 20—30 minutes. In 41 cases subjected to ischemia for 15 minutes, neither paralysis nor paresis was observed in the hand or the muscles of the arm. In those cases (about 10) where the sensibility was investigated, all that was found was a feeling of numbness, involving the two outermost phalanges of the finger.

The figures for the time taken to bring about muscle paralysis in the five tetany cases are given in table XVII. In these cases too the anesthesia was tested with a piece of cotton-wool.

Table XVII.

Time taken to produce ischemic motor and tactile paralysis in tetany cases.

Case	Motor paralysis	Anesthesia
I.	12′ Total motor paralysis of fingers and wrist	12′ up to the middle of the dorsal side of the forearm
II.	13′ Total motor paralysis of fingers and wrist	13′ up to the middle of the dorsal side of the forearm
III.	7′ Discontinued because of pain	—
VI.	16′ Paralysis of the small hand muscles	15′ up to the middle of the dorsal side of the forearm
V.	16′ Total motor paralysis of fingers and wrist	16′ up to the wrist

As will be seen from the table, the paralysis both of the motor and sensory fibres is accelerated. A deficiency of ionized calcium thus considerably intensifies the sensitiveness to ischemia. This may be explained, at any rate in part, by the greater requirement of oxygen entailed by the increased activity in the sensory and motor fibres. Summing up, it may be stated that the dimi-

nution of ionized calcium augments the spontaneous nerve activity occurring during and after ischemia. At the same time the nerve is more easily paralyzed by ischemia.

4. The nervous mechanism of the typical symptoms of irritability.

The mechanism of the symptoms of irritability in tetany has, in the course of years, been dealt with in several investigations by various authors. Most of these investigations have been made on animals suffering from experimentally induced tetany. The symptoms of tetany vary in different kinds of animals (DRAGSTEDT, 1927), and the forms of the disease also deviate somewhat from those in human subjects. The principal symptoms, however, are the same: the spasms, the fasciculations and increased electric excitability of the nerve. There is every reason to conclude that the mechanism in man is the same as in the laboratory animals, cats and dogs. The investigations made on animals are, however, partly conflicting.

In man the different symptoms of irritability in a tetany attack typically occur in the following sequence: first "tingling", and then the typical tetany spasm, with an intense sensation of tension in the affected muscles. As pointed out in Chapter 2, there is a certain lack of correspondence between the spastic sensation and the objective symptoms of spasm, the feeling of tension being considerably intenser than might have been expected from the slight, at first barely perceptible, spasm. Fasciculations may occur, and if they do so, usually appear in close connection with the spasms. In man, however, as compared with cats and dogs, this symptom is but little marked. Concurrently with the symptoms of irritability, the electric excitability in the nerves is increased. The entire sequence of symptoms has been studied in this paper, whereas previous investigators have dealt with the spasms, the fasciculations and the changes in electric excitability only. A brief description of these investigations will now be given.

Electrical excitability. The earliest and most constantly occurring phenomenon observed in experimentally induced tetany in animals has been found to be an increase in the electrical, and to some extent in the mechanical, excitability of the motor nerves. The electrical

excitability has been measured as the thresholds for the cathodal and anodal closing and opening contractions. However, it was not found possible to refer any nervous symptoms to the increase of the excitability thus tested. Indeed, PATON, FINDLAY and WATSON (1916) state that the absence of direct relationship between the severity of the nervous symptoms and the electrical excitability of the peripheral nerves clearly shows that this altered excitability is of subsidiary importance compared with the changes in the central nervous system. The increased excitability of the nerves is independent of the central nervous system, seeing that, as shown by MACCALLUM (1913), PATON and associates (1916), WEST (1935), etc,. it continues after section of the nerve. As MACCALLUM showed by perfusion of the isolated leg of a normal dog with blood from another tetany dog, it is due to the direct action of the blood on the peripheral nerve. According to PATON and co-workers, the nerve-endings appear to be the point acted upon, seeing that in cross-circulation experiments, the result of stimulation of the sciatic nerve bathed in the animal's blood varies according to the changes produced in the peripheral nerve supplied with the foreign blood.

Fasciculations and tonic spasms. In regard to the mechanism of the motor symptoms of irritability, MUSTARD (1911) states that ablation of the motor cerebral cortex in parathyroidectomized dogs has no effect on the symptoms. PATON and associates (1916) maintain that, whereas the cerebral arch is not directly involved in the production of spasticity tremor and jerkings, the integrity of the cerebellar arch is essential for the spastic tone. SPIEGEL and NISCHIKAWA (1923), on the other hand, contended that ablation of the cerebellum had no effect, and located the centre of the tonic spasms in the *pons* and *medulla oblongata.*

CARLSON and JACOBSON (1911), as also MUSTARD (1911), found that on high thoracic transection of the spinal cord in parathyroid tetany, the symptoms posterior to the lesion disappeared. According to LUCKHARDT, SHERMAN and SERBIN (1920), on the other hand, fibrillary contractions and clonic spasms still occur posterior to the transection. The tonic spasms are less pronounced anteriorly. They further noted, in parathyreoprivic dogs, a great increase in the reflex irritability during tetany attacks. WEST (1935) found that transection of the cord had no effect on the symptoms distally thereto: tonic, clinic and fibrillary tetany continued.

According to PATON and associates, section of the nerves abolishes all spasm, tremor and jerkings. WEST (1935), on the other hand, states that fibrillary tetany continues after nerve section; and that dorsal root section abolishes "tonic" tetany, which is thus dependent on the integrity of the lowest reflex spinal arch. From these experiments he inferred that parathyroid tetany arises essentially from the action in the blood directly on the muscle, which gives rise to fibrillary tetany and raised electrical excitability. Given the integrity of the spinal reflex arch, the individual muscle contractions are synthesized into tonic spasms. Other authors, such as FALTA (1928), locate the origin of the paresthesias and spasms in the *medulla spinalis* and *oblongata.*

Some further investigations have been made on human subjects. SCHÄFFER (1920) recorded the muscle action potentials in the spastic muscle on the occurrence of the Trousseau phenomenon. He found that the potentials had the same frequency and character as is typical of voluntary contraction. From this he inferred that the Trousseau spasm is maintained by impulses proceeding from the centre.

BEHRENDT and FREUDENBERG (1923) studied the spasms in hyperventilation tetany. They found that the Trousseau phenomenon could be produced in the arm after anesthesia of the arm plexus with novocain. Such anesthesia of the *nervus medianus*, *ulnaris* and *radialis* at the wrist did not prevent the occurrence of spasm in the paralyzed hand. But in one, at any rate, of the two reported experiments, the typical hand posture did not occur, but a maximal flexion of the finger instead of extension of the end phalanges.

DITTLER and FREUDENBERG (1923) took records of action potentials in the hand muscles: they found that spasms in the hands might occur without the action of such impulses. From this BEHRENDT and FREUDENBERG conclude that the spasm is not maintained by impulses from the motor paths, but with the aid of special parasympathetic "tonus fibres"; and that the blood changes which produce the spasm act directly on the *nerve-endings*, seeing that, if novocain is injected, instead, into the muscle itself, it considerably reduces the spastic tendency.

DITTLER and FREUDENBERG subsequently (1924) controlled the novocain experiments, in view of the possible error that the small muscles of the hand, when records were taken of the action potential, might not have taken part in the spasms. The spastic posture of the hand, they thought, might be caused solely by the long muscles of the fingers, which were not paralyzed by the novocain. This was indicated by the atypical attitude of the hand.

The results of these renewed tests were partly conflicting. We must therefore for the present leave open the question whether the tests conduce to substantiate the probability that spasm without action potentials really exists, or whether the tetany spasm can occur at all when the motor fibres have been blocked.

It has also been discussed whether the Trousseau sign is a purely peripheral phenomenon, as was supposed by Trousseau himself as well as by THIEMICH and others, or merely a reflex, as contended by FRANKL-HOCHWART and SCHLESINGER (cited by NOTHMAN 1937).

a. The electrical excitability.

That the excitability in the nerve endings should determine the excitability of the whole nerve, as PATON and co-workers seem to consider, is scarcely probable in view of the experiment reported on p. 66. In that test the electrical excitability in the peripheral part of the nerve could, by means of ischemia, be caused to vary within wide limits without any modification of the excitability in the proximal non-ischemic part of the nerve. In order to settle

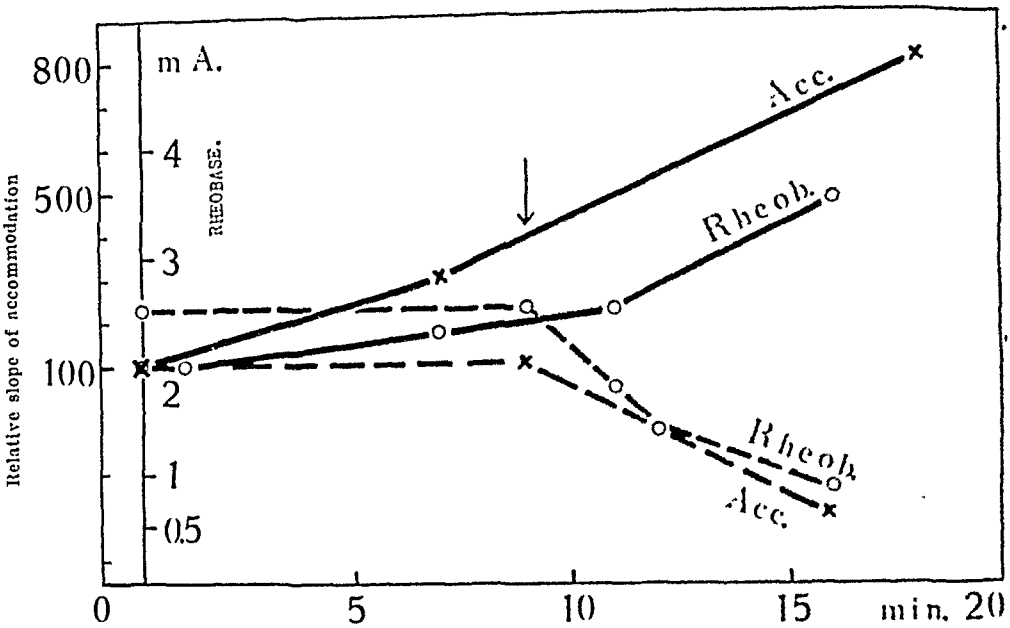

Fig. 18. The changes in rheobase and accommodation proximally (broken line) and distally (solid line) to a pneumatic cuff distended above the systolic pressure during hyperventilation. The hyperventilation commences at the point indicated by the arrow. The rheobase is given in the absolute value, the accommodation in the relative.

this question as regards human subjects, tests were made in which the excitability was determined firstly at the wrist, and secondly at the middle of the upper arm on the fibres of the ulnar nerve to *musc. inteross. dors.* 1. A pneumatic cuff was distended somewhat proximally to the elbow between the electrodes. It was kept on for 9 minutes in order to give time for the subsidence of the increased excitability (in the ischemic part of the nerve), which at any rate comprises the rheobase. The forced respiration then commenced. See Fig. 18.

Owing to the arrest of the blood-flow to the periphery, the blood changes induced by hyperventilation cannot affect the nerve-endings nor the distal part of the nerve. As shown by the above Figure, the rheobase rises, and the accommodation increases, at the wrist, in spite of the ischemia. Proximally to the cuff, on the other hand, the rheobase and accommodation values fall, just as in the hyperventilation tests without a pneumatic cuff. From this we seem to be warranted in inferring that *the excitability of the nerve is not determined by the nerve-endings.* The excitability can

evidently be modified in different parts of the nerve independently of one another.

In regard to other questions bearing on the excitability, the reader is referred to the preceding chapters.

b. The typical form of tingling in tetany.

As was shown in chapter 1 and 2, the typical form of tingling in tetany is a tactile paresthesia. In order to ascertain where the impulses which give rise to this paresthesia actually originate, the following experiment was made by the author on himself. It is clearly described in the following protocol.

Experiment A.

Subject: Author, 10th February 1944.

0′ A pneumatic cuff is distended to 250 mm somewhat *proximally to the elbow* of the right arm. After 2 minutes a tactile paresthesia commences in the thumb and forefinger of the right hand. The paresthesia has cleared away after 6 minutes.

6′ Forced respiration begins.

8′ *Tactile paresthesia begins simultaneously in the fingers of both hands*, and spreads up over the hand. The intensity is almost the same in both hands, though possibly somewhat greater in the right hand.

10′ The forced respiration is discontinued. The paresthesia immediately begins to subside and ceases simultaneously on both sides at 15′.

9th Feb. 1944. The same experiment is repeated with the cuff placed on the left arm. Results as above.

Experiment B.

On the author himself, 8th February 1944.

0′ The pneumatic cuff is distended to 200—250 mm *as high up as possible round the right upper arm*. After 7 minutes the tactile paresthesia which had set in has cleared away.

7′ Forced respiration begins.

9′ *Marked tactile paresthesia in the fingers of the left hand, spreading over the hand.*

11′ *Paresthesia begins in the right hand, but is much less marked than in the left hand.*

13′ Forced respiration is discontinued. The paresthesia first clears away in the right hand.

7th Feb. 1944. The same experiment was made with the cuff on the left arm. The paresthesia begins later and is less marked in the left arm.

Experiments A. and B. were repeated on two other persons, with the same results.

Experiment A. shows that the most distal portion of the nerves in the region where the paresthesia is perceived plays scarcely any part in the production of the paresthesia, seeing that no difference is observed if they are secluded from the effect of the blood changes due to the forced respiration.

Experiment B. shows that the impulses which give rise to the paresthesia proceed most readily and in the main from the stretch of nerve peripheral to the axilla, but may also originate proximally to it. Thus the tactile paresthesia felt in the arm is caused by impulses proceeding primarily from the longest tactile fibres (seeing that the paresthesia is located in the hand) between the elbow and the axilla. It is evidently first and foremost a peripheral phenomenon. ZOTTERMAN (1933) also states that, on hyperventilation, the neurons with long axons are first affected.

c. *The spasms and the sensation of spasm.*

The typical tetany spasm in the small muscles of the hand was studied with the same technique as that adopted for the analysis of the paresthesia. The experiment is described in the following protocol.

Experiment A.

N-m, aged 21, 10th February 1944.

0′ A pneumatic cuff is distended immediately *above the right elbow.* Forced respiration begins.

2′ Slight feeling of tension in the right hand, immediately followed by a similar feeling in the left.

3′ *The tension passes into a typical spastic attitude of both hands.* Forced respiration is continued for 10 minutes. Spasms in both hands, but somewhat more marked in the right.

Experiment B.

N-m, aged 21, 9th February 1944.

0′ A pneumatic cuff *as high up as possible* round the *left upper arm.* Another cuff round the *right arm somewhat proximally to the elbow.* Distended to about 250 mm. Forced respiration begins.

2′ Feeling of tension in the right hand.

3′ Spasm in the right hand.

10′ Contractions in the right hand and wrist, which cannot be voluntarily mastered. *In the left hand neither tension nor contraction.* Forced respiration ceases.

8th Feb. 1944. The same experiment, but with the position of the pneumatic cuffs reversed. Spasms in the left hand, but none in the right.

These experiments show, as in the case of tactile paresthesia, that the peripheral stretch of nerve, the nerve-endings and the muscle do not play any noteworthy part in the production of the spasms, it being possible to provoke them even if the periphery is secluded from the effect of the blood changes. They also indicate that the impulses which produce the spasms proceed primarily from the stretch of nerve between the elbow and the axilla.

Experiment C.

Subject: Author, 11th February 1944.

0′ A pneumatic cuff *round the left arm somewhat proximally to the elbow* is distended to 200—250 mm.

9′ Forced respiration begins.

10′ Tactile paresthesia begins in the fingers of the left hand, immediately followed by those of the right.

15′ Feeling of tension in both hands, with the impression that they are striving to assume the typical spastic attitude.

17′ In both hands contractions which can scarcely be voluntarily mastered. Intense feeling of tension.

20′ The spasms begin to subside in the left hand, whilst capacity for voluntary contraction diminishes.

25′ The spasms have ceased in the left hand, concurrently with complete paralysis of the voluntary movements of the fingers. Intense feeling of tension in both hands. Spasms as before in the right hand.

36′ *Paralysis of the movements of fingers and wrist.* Anesthesia up to the middle of the back of the hand, *suspended feeling of movements of the wrist and fingers of the left hand. But intense feeling of tension,* plus tingling, in the hand. In the right hand spasms as before. The forced respiration is discontinued.

This experiment invites to the following conclusions:

1. That spasms and voluntary movements are produced by impulses transmitted through the same fibres, as both are simultaneously paralyzed by ischemia. This argues against the theory of FREUDENBERG and associates, which, from many points of view, seems untenable, that the spasm is maintained by special "tonus" fibres.

2. The sensation that the hand muscles are strongly contracted is not induced by the muscle spasm, seeing that it is felt as intensely despite the fact that the muscle has been paralyzed and is quite limp. The feeling of tension is thus induced by an inadequate

stimulus, and is consequently a paresthesia. As indicated by the results of experiments A. and B., the impulses which give rise to the feeling of tension proceed mainly from the stretch of nerve between the elbow and the axilla. This sensation was felt as intensely as before if the peripheral part of the nerve was secluded from the blood changes, but was at first suspended if the stretch of nerve between the elbow and the axilla was withdrawn from the circulation. The fibres which induce the feeling of tension are presumably muscle afferents belonging to the muscle spindle apparatus.

d. The Trousseau sign.

This phenomenon has been studied in the same way as the tactile paresthesia induced by ischemia (see p. 70). A pneumatic cuff is placed and distended on the forearm. After the lapse of a few minutes, spasms and a feeling of tension ensue, but, when the excitability due to the continued ischemia has diminished, these symptoms pass away. A second cuff is then distended proximally to the first one. The same phenomena thereupon recur, though the spasms become much more intense. Finally, a third cuff is distended as far proximally as possible (without removing the first and second). It is then found that the Trousseau sign appears soonest at that point and has the greatest intensity there, just as was the case in tactile paresthesia. This indicates that the phenomenon is not produced at the periphery, but by ischemia acting on *the nerve trunk itself*, and the more readily the further *proximally* the compression is made on the arm. This is evidently due to the fact that (as shown by LEWIS and associates) the ischemia affects the nerve mainly in its proximal part, where the excitability is also most increased by the deficiency of ionized calcium.

Evidently the stimuli which give rise to the Trousseau sign are most readily produced from those parts of the nerve where the impulses which cause the spontaneous "calcium-deficiency spasm" and the feeling of tension derive their origin. Here the ischemia thus provokes a spontaneous activity in the fibres of the muscle proprioceptors, which produces that feeling of tension and probably also the spasm (see the next chapter). This, however, is not at all surprising. As previously indicated, a spontaneous activity in the longest tactile fibres which results in the tactile paresthesia is *normally* observed during ischemia. It is identical with the "cal-

cium-deficiency" paresthesia. It might be termed a sensory Trousseau sign — as far its mechanism of origin is concerned —, but, as just indicated, it is quite a normal phenomenon. When the ionized calcium is reduced, the tactile paresthesia increases in range and intensity. Not only the longest tactile fibres, but also the shorter ones begin to discharge spontaneously. The ischemia and the deficiency of ionized calcium thus mutually reinforce one another and unite in producing the same effect. This gives rise to a spontaneous activity also in other fibres (such as the short tactile fibres and those of the muscle proprioceptors), which normally do not discharge spontaneously, under the influence of ischemia.

5. Summary and Conclusions, Section V.

In tetany, the rheobase is lowered and the capacity of the motor nerve for accommodation is reduced. It has been shown in regard to the ulnar nerve that these *changes in the electrical excitability* are more pronounced in the *proximal* part of the nerve at the axilla than distally at the wrist.[1]

It has further been shown that the changes in the electrical excitability of the nerve occur independently of any such changes at the periphery of the nerve and in the nerve-endings. In fact, during forced respiration, quite as marked lowering of the rheobase and slowing-down of the accommodation ensue in the proximal part of the nerve despite the arrest of the blood-flow to the periphery of the limb, whereas the excitability of the nerve induced by ischemia moves in the opposite direction. We have thus found that the excitability can be changed in the different parts of the nerves independently of one another and of the nerve-endings.

It has also been noted (see the historical section) that the frog nerve, on reduction of the ionized calcium, begins to discharge spontaneously, a phenomenon which is closely connected with a lowering of the rheobase and with poor or non-existing capacity for accommodation. Attention has been drawn to similar observations made by LEHMANN (1937 b) in regard to the mammalian A nerve fibres *in vitro*.

In the author's experiments with hyperventilation tetany,

[1] In the section on tetany caused by forced respiration and in the report on clinical case No. 1 with hypoparathyreoidism.

and, as shown in his clinical case No. 1, there is a close connection between the diminishing capacity for accommodation and the occurrence of symptoms of irritability. In the proximal part of the ulnar nerve, though not in the distal, the accommodation was almost non-existent and the rheobase, as measured in the fibres of the hand muscles while fasciculations were proceeding there, was remarkably low. It is therefore very probable that in these cases the *fasciculations* were caused by a spontaneous activity in the proximal parts of the longest motor fibres in the arm, as was shown to be the case in regard to the type of fasciculations occurring after a sufficiently lengthy ischemia in the same parts of the nerve (p. 67). Both types of fasciculations have also in other respects essentially the same character.

The paresthesias. The typical "calcium deficiency" paresthesia (in clinical cases 2, 4 and 5 and in all the hyperventilation tests) has been shown to be identical with that produced by ischemia (p. 69). It is thus a tactile paresthesia. As the paresthesia diminishes the appreciation of light touch, it is naturally regarded as a feeling of numbness. Some of the persons who took part in the experiments with forced respiration in fact volunteered the remark that they felt a sensation of numbness, and they did not notice the tingling itself until questioned on the subject. This form of tingling is therefore doubtless largely responsible for the numbness, which is a common symptom in tetany. Another reason for the frequent occurrence of the feeling of numbness as a symptom is that the nerve is paralyzed by ischemia much more rapidly than normally. The woman in clinical case No. 5 spontaneously remarked that, when she sat with her legs crossed or lay in such a position that pressure was exerted on the nerves of her arm (flexing of the elbows etc.), her legs and arms got numb more rapidly *after* her illness than before it.

It has further been demonstrated that the impulses which cause the tactile paresthesia originate in the peripheral nerves: in the arm primarily in those parts of the nerves which lie between the elbow and the axilla; but that the focus seems gradually to spread proximally thereto. Nevertheless, if the effect of the blood changes was sufficiently marked, the entire peripheral nerve would doubtless give rise to spontaneous activity. There is no reason to suppose that such impulses could not be set up also along the central tactile paths. The paresthesia, however, began in the stretch of nerve between the elbow and the axilla. Since the feel-

ing of tingling began at the periphery and spread centripetally, the touch fibres from which that sensation ultimately proceeded must be the longest ones.

The feeling of tension and the spasm itself. It has been shown that the *sensation of tension* is not caused by the actual muscle spasm, but is produced by impulses which come from the nerve itself, and primarily from the stretch of nerve between the elbow and the axilla. We are thus concerned with a paresthesia presumably proceeding from the fibres of the muscle proprioceptors. The impulses which cause *the spasms* do not come from the muscle itself, nor from the nerve-endings, but from that very part of the nerve where the impulses which produce the tactile paresthesia and the feeling of tension derive their origin. The motor impulses probably pass along the fibres concerned in voluntary movements, seeing that these movements are paralyzed by ischemia concurrently with the production of spasms.

If these findings are correlated with the pertinent observation made by WEST (1935), in experiments on parathyroidectomized dogs, that the spasms cease on severance of the posterior roots, it seems plausible to suppose that the spasms are caused by the spontaneous activity in the fibres of the muscle proprioceptors, which reflexively maintain the spasms. Whether this reflex action also involves a pathological increase of irritability in the *medulla spinalis*, or not, could not be determined with the technique adopted here.

Assuming that the changes in electric excitability are of the same character in the sensory nerve fibres as in the motor fibres — as shown by HOFFMAN as far back as 1888 in regard to the rheobase — the mechanism of the phenomena here referred to may be briefly described as follows:

The deficiency of ionized calcium tends to lower the rheobase and to slow down the accommodation in the nerve. In the arm these changes lead to spontaneous discharges primarily in the proximal part of the longest nerve fibres, from which the focus spreads centripetally and centrifugally. The spontaneous activity starts in the tactile fibres from which the typical sensation of tingling originates, and somewhat later is set up in the fibres of the muscle proprioceptors, which produce the feeling of tension and, reflexively, the spasm itself; finally it reaches also the motor fibres, giving rise to fasciculations.

It has further been pointed out how the localization of the

spontaneous activity in cases of calcium deficiency corresponds with the region showing the greatest sensitiveness to ischemia. As demonstrated by LEWIS and co-workers (1931) and ZOTTERMAN (1933), the ischemic paralysis is most liable to affect the fibres in the proximal part of the arm. Here too the longest tactile fibres are first involved and afterwards the longest motor fibres and the sense of muscular tension.

It might be asked, as in the case of ischemic paralysis, whether the localization of the calcium deficiency symptoms and the order in which the different fibres set up spontaneous activity is not directly dependent on their calibre. However, in regard to the calibre of different parts of the nerve as well as of the different long and short fibres, and probably also as regards the order of sequence between the coarsest tactile fibres, the muscle afferents and the motor fibres, our knowledge is too imperfect to permit any definite statement to be made on the subject. The calcium deficiency symptoms, however, evidently proceed from the coarse fibres.

The close connection between ischemia and changes in the amount of ionized calcium is indicated by the previously noted fact that calcium deficiency accelerates ischemic paralysis, besides which the ischemia aggravates the symptoms of irritability entailed by a deficiency of ionized calcium, thus giving rise not only to the Trousseau sign, but also to the increased intensity and spread of the tactile paresthesia.

General Summary.

The results set forth in this work have been separately summarized at the end of each section. This general review is intended merely to serve as a broad survey, the reader being referred to the separate summaries for a more detailed recapitulation.

The neglected subject of nerve accommodation in man, in relation to the general problem of nerve excitability, has been studied with a method for quantitative analysis by means of an apparatus for producing exponentially rising, stimulating currents. With this method, accommodation curves can be measured with a standard error of ± 5 %.

Section III gives an analysis of the normal variations of accommodation in man. These are relatively small: in a single individual, repeated determinations over long periods range between 6 and 8 per cent. of the average value. Comparisons have been made between motor nerve, motor point, different motor and sensory nerves, etc. A detailed summary will be found on page 50.

The effect of ischemia upon rheobase and accomodation has been studied with the aid of pneumatic cuffs. Considerable changes in accommodation could be shown by this method. They were found to be most marked in the proximal portion of the longest nerve fibres of the arm. It was found possible to correlate changes in excitability, as measured by our method, with symptoms of irritability, such as fasciculations and paresthesias, and to show that they were due to spontaneous activity in nerves, characterized by a low rheobase and poor or absent accommodation.

Section IV is devoted to a description of the effects of such artificially induced circulatory disturbances upon the accommodation. The results are summarized on page 75.

The capacity for accommodation was found to be reduced in 4 out of 5 clinical cases suffering from tetany.

Artificially induced tetany was studied with the aid of hyperventilation. Here too a reduced capacity for accommodation was

regularly observed, especially in the proximal portion of the arm nerves. Spontaneous activity, starting in the longest motor fibres of this region, gave rise to fasciculations in the muscle.

By combining artificially induced tetany with ischemia, it was found possible to analyze the typical symptoms of tetany, such as various paresthesias, tetanic contractions and the Trousseau sign, all of which manifestations were found to be connected with spontaneous activity in the proximal region of the longest fibres of the arm. One type of paresthesia was found to be localized in the fibres transmitting light touch, whilst another type of paresthesia, namely the sensation of tension in the muscles, as well as the actual spasms and the Trousseau sign, was caused by spontaneous activity originating in the muscular afferents; the fasciculations in the muscles could be ascribed to similar activity in the motor fibres.

Ischemia and calcium deficiency were found to have similar effects, either of them tending to aggravate the symptoms caused by the other. Moreover, in both cases, the proximal portion of the longest nerve fibres of the arm is the most sensitive, and the different nerve fibres are involved in the same order. The symptoms of calcium deficiency primarily originate in the coarse nerve fibres. A detailed summary of these results, described in Section V, will be found on page 97.

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STUDIES ON MUSCLE

By

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The Editor.

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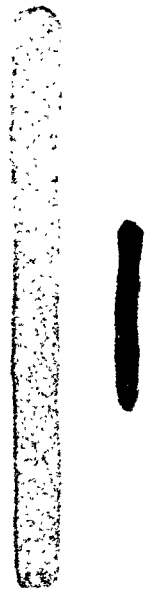
Abbreviations.

DTP: Adenosine-triphosphate or adenyl-diphosphate.
ADP: Adenosine-diphosphate.
AMP: Adenosine-monophosphate or adenylic acid.
DR: Double Refraction.
DRF: Double Refraction of Flow.

Introduction.

Motion has always been looked upon as the index of life. The organ of motion is muscle. If the muscle fibre is treated with an alkaline salt-solution, water insoluble proteins are extracted which can be brought into the form of a fibre again (Fig. 1 a). If such



a. b.

Fig. 1. a. Actomyosin thread (myosin B). b. The same thread contracted. Magn. 1: 30.

a fibre is suspended in a fresh watery muscle-extract, it contracts energetically (Fig. 1 b). The vital function of muscle is thus reproduced *in vitro* in a system, built of constituents of muscle. The present book is devoted to the analysis of this phenomenon.

Circumstances did not permit working out all points as was desired. That my literature of the last years is incomplete, is no more than natural.

Most of the work to be reported here is laid down in the four consecutive volumes of the Studies from the Institute of Medical Chemistry, University Szeged, S. KARGER, Basel, New York, the fourth volume of which remained, owing to circumstances, unpublished. It is mostly the work of my pupils and collaborators. So if I ornate myself with feathers that are not mine, I may be excused by the fact that the birds all belong to my own poultry-farm.

PART I.

Myosin.

History.

Striated muscle contains about 20 % of protein. If the muscle is minced and extracted with water, half of this protein goes into solution. This soluble muscle-protein has been called by v. FÜRTH "moygen".

The insoluble fraction contains several enzymes; involved in oxidation, like succinodehydrogenase, citricodehydrogenase, cytochromoxidase, diaphorase and the cytochromes. In this insoluble fraction we also find the contractile substance of the fibril. This insoluble protein-fraction attracted the attention of early biochemists like DANILEWSKY, HALLIBURTON, and v. FÜRTH, but it was not until thirty years later that its study was taken up more in detail.

H. H. WEBER extracted from this water-insoluble fraction of muscle a globulin-like protein which could be precipitated by diluting the salt-solution, used as a solvent. This protein made up 40 % of the total amount of protein, it seemed to be the main constituent of the fibril and to be identical with v. FÜRTH's "myosin". WEBER, in collaboration with G. BOEHM, K. MEYER, R. STÖVER, F. KAMP and W. HOLLWEDE, made myosin the object of his very careful and extensive investigations.

The next researcher to give his full attention to myosin was J. T. EDSALL who studied the general properties of this protein very thoroughly, while A. v. MURALT and J. T. EDSALL studied the DRF of myosin and made observations on its viscosity.

Myosin, having thus been recognised as an individual protein, occupied a great number of research workers. The isoelectric point of myosin was the object of much discussion. H. H. WEBER (70), following the migration of myosin particles in the electric field,

found the I. P. at pH 5.1. At this pH lay also the minimum of swelling of the insoluble muscle residue. J. B. COLLIP and W. T. SALTER, however, found the flocculation-optimum between pH 6.2—6.6. SALTER found the pH of well washed myosin in the same region. According to EDSALL the minimum of acid-base-binding capacity also lies at this pH. WEBER corroborated these observations but, in collaboration with HOLLWEDE, expressed the opinion that EDSALL worked with alkali-myosinate, and not with free myosin; the I. P. of free myosin is at pH 5.3.

E. C. BATE-SMITH (5) reinvestigated the question and DUBUISSON (16, 17) made careful measurements on the acid-base-binding power of myosin.

The solubility and extraction of myosin were studied by HOWE, SALTER, WEBER, EDSALL, WEBER and MEYER, SMITH, HENSAY, and GREENSTEIN and EDSALL.

The amino-acids of myosin were studied by SHARP, TODRIK and WALKER, and BAILEY. Comparative chemical studies were made by BAILEY and MEHL.

The SH of myosin was the object of the studies of MIRSKY, GREENSTEIN and EDSALL, GREENSTEIN and MEHL, chiefly in relation to the denaturation of myosin. D. M. NEEDHAM studied SH in relation to enzymic activity.

DEUTICKE observed the rapid decrease of solubility of myosin after muscular activity and *post mortem*. These changes were the object of the studies of HENSAY, MIRSKY (45) KAMP, SMORODINTSEV and BYSTROV. E. C. SMITH (65) made the disappearance of a labile substance responsible for the rapid post mortal changes. D. GEBERS found that ATP increased the solubility of muscle-proteins, as well in fresh resting muscle as well as in muscle after work or storage. MIRSKY (44) showed that myosin becomes insoluble in monojodoacetate poisoning.

MIRSKY (43, 44, 45), SAXL, DUBUISSON (15), DUBUISSON, LECOMTE and MONNIER, WESTERBRINK and KRABBÉ tried to correlate the physicochemical properties of myosin with the different physiological conditions and the function of muscle.

Myosin threads were studied, following the method of WEBER (74), by DUBUISSON (18), DUBUISSON and MONNIER.

The binding of K by myosin was studied by C. MONTIGEL, who found that the myosin could bind 0.62 % K very firmly. The K-binding capacity was diminished by choline and acetylcholine, ATP and ADP.

Among different physical properties the UV spectrum was studied by M. N. LJUBIMOWA and M. S. SCHIPALOW, J. W. MEHL tried to determine the dimension of the myosin particle; BOEHM and SIGNER gave evidence about its long, threadlike shape. The MW was measured by WEBER and STÖVER, who found it about 10^6. Urea disintegrated myosin into particles of about 10^5. E. GORTER and H. v. ORMOND studied the spreading of myosin on water-surfaces. BATE-SMITH (5) presented a more general study of the physical properties of myosins. THE SVEDBERG, using the ultracentrifuge, found myosin unstable and heterodisperse.

The polysaccharide compounds of myosin were studied by ST. J. PRZYLECKY and his collaborators R. MAJMIN and P. FILIPOWICZ.

General attention turned towards myosin when ENGELHARDT and his collaborators MEITIANA and LJUBIMOWA found a direct relation between myosin and ATP. The splitting of ATP is, according to our present knowledge, the source of the energy of muscular contraction. ENGELHARDT and MEITIANA found that among the number of substances studied, ATP only had a definite influence on the physical properties of myosin, if it was applied in small concentration: it increased the extensibility of myosin threads.

It has been known for some time that muscle is capable of splitting off two phosphate groups from ATP. K. LOHMANN found that the water-insoluble fraction of muscle was capable of splitting off one phosphate, while the splitting off of the second phosphate could be activated by Mg. ENGELHARDT and LJUBIMOWA stated that the splitting off of the first phosphate could not be separated from myosin and that this activity, in different preparations, went parallel to their myosin-content. They concluded that it was myosin itself which was responsible for this splitting. The splitting off of the second phosphate was due to a water-soluble substance since, by repeated washing, myosin lost the power of attacking the second phosphate.

J. NEEDHAM, SHIH-CHANG SHEN, D. M. NEEDHAM and A. S. C. LAWRENCE found that the viscosity and DRF of myosin was, under certain conditions, decreased by small amounts of ATP in a reversible way. In a later paper by J. NEEDHAM, A. KLEINZELLER, M. MIALL, M. DAINTY, D. M. NEEDHAM and A. S. C. LAWRENCE these observations were extended: the same effects were produced by inosinic triphosphate and ADP. Inorganic triphosphate was inactive but completed with ATP.

BANGA and myself (108) found that, according to the duration of extraction, myosin could be prepared in two different forms: short extraction yielded the liquid "myosin A", prolonged extraction the very viscous "myosin B". Threads, prepared from myosin B, if suspended in a freshly prepared watery extract of muscle, or a solution containing K, Mg and ATP in the same concentration, gave a violent contraction (Fig. 1), while threads of myosin A were relatively inactive. The low viscosity of myosin A solutions changed only slightly on addition of ATP while myosin B, if dissolved in 0.6 *M* KCl, responded to the addition of ATP by a great fall of its viscosity. This fall of viscosity was termed "activity" and "100 % activity" was called the maximum activity of myosin B. Thus, by comparing it with myosin B, the activity of any preparation could be expressed numerically.

This opened the way to the quantitative study of the myosin A-B transformation. This work was undertaken by F. B. STRAUB who made the discovery that the transformation of myosin A into myosin B was due to a second protein which is brought into solution on prolonged extraction. He called the new protein "*actin*". Actin and myosin unite to form the highly viscous "actomyosin". In presence of high salt-concentrations this actomyosin is dissociated by ATP into its components again, hence the fall of viscosity (activity).

Conclusions could be drawn from the "activity" on the actin-content of actomyosin. Another quantitative expression was thus introduced: "% actomyosin" by which the quantity of actin, present in 100 parts of actomyosin, was meant. The "100 % active" myosin B was found to be a 16.7 % actomyosin, a myosin, 100 parts of which contained 16.7 % of actin. "Myosin A" is an actomyosin of undefined, but low actin content.

These quantitative methods opened the way to the purification of myosin and actin and lead eventually to the crystallisation of myosin and the preparation of pure actin.

The properties of actin-free myosin widely differ from those of actomyosin. Even such small admixtures as 1—2 % of actin greatly modify the physical and chemical properties of myosin. Earlier authors worked invariably with myosin, more or less heavily contaminated with actin. What is described as "myosin", is but an impure actomyosin of unknown composition. In some instances the properties, described as typical properties of "myosin" were rather the properties of the actin than those of myosin.

So, for instance, v. MURALT and EDSALL wanted to reserve the name "myosin" for the substances showing DRF in presence of higher salt-concentrations. Myosin, under these conditions, has no DRF at all, contrary to the actin which shows a brillant DRF. It is thus evident that most of the data of the literature, relating to myosin, will need revision. For this reason I have refrained from their detailed discussion.

Lately SCHRAMM and WEBER have found by ultracentrifugation that "myosin" is composed of two different, but in themselves homogeneous fractions, one of which has a high and the other a low speed of sedimentation. It seems not unlikely that their "light myosin" is myosin, and that their "heavy myosin" is actomyosin.

Using the electron-microscopic method ADRENNE and WEBER found several thousand Å long and 5—10 Å wide threads in "myosin". It seems possible that they were actomyosin micels, while the breaks in these threads corresponded to parts of the actin thread uncoated by myosin.

Some properties and reactions of myosin.

Myosin crystallises in the form of very fine needles (Fig. 2) with a strong tendency of lateral association. Needles, observed with high power, will often be found to be bundles of finer needles. (Fig. 3.) Sometimes the needles associate to fine, long threads, fibrils. (Fig. 4.)

Analysis. Recrystallised myosin was precipitated with alcohol and extracted with boiling abs. alcohol for two hours. The dried material was analysed and showed the following composition:

C	50.04 %
H	7.70 %
N	16.15 %
S	1.14 %
Ash	1.23 %

These data agree with the composition of a protein. According to the analysis myosin contains 6 S for 17,000 g. Since the analysis of S involves some loss, this indicates that 17,600 g. contain 6 S atoms, and myosin, similarly to other proteins, is built of such units.

The analysis of the alcoholic extract showed that recrystallised myosin contains 3 % lipoid matter, calculated for dry weight. This lipoid is partly insoluble in acetone.

Free myosin, being soluble in water, cannot be classed among

Fig. 2. Myosin crystals. Magn. 1: 90.

globulins. On the other hand, on adding ammonium sulphate, myosin precipitates as we pass half saturation.

Stability. Myosin, if dissolved in 0.5 *M* KCl and neutralised, can be kept at 0° C without loss of enzymic activity for a fort-

Fig. 3.

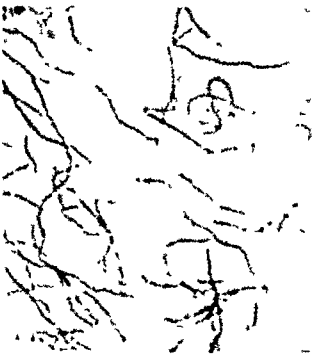


Fig. 4.

Myosin threads. Magn. 1: 40.

night. Its finer colloidal properties, however, change after a few days storage: its reactions become sluggish, its viscosity and viscosity-anomaly rises. As will be shown later, the metal-binding capacity, which governs all these reactions, is appreciably decreased even after 24-hours storage at 0°.

Incubation of myosin (dissolved in neutral 0.5 *M* KCl) for ten minutes at 37° causes not only rise of viscosity but even drop of enzymic activity.

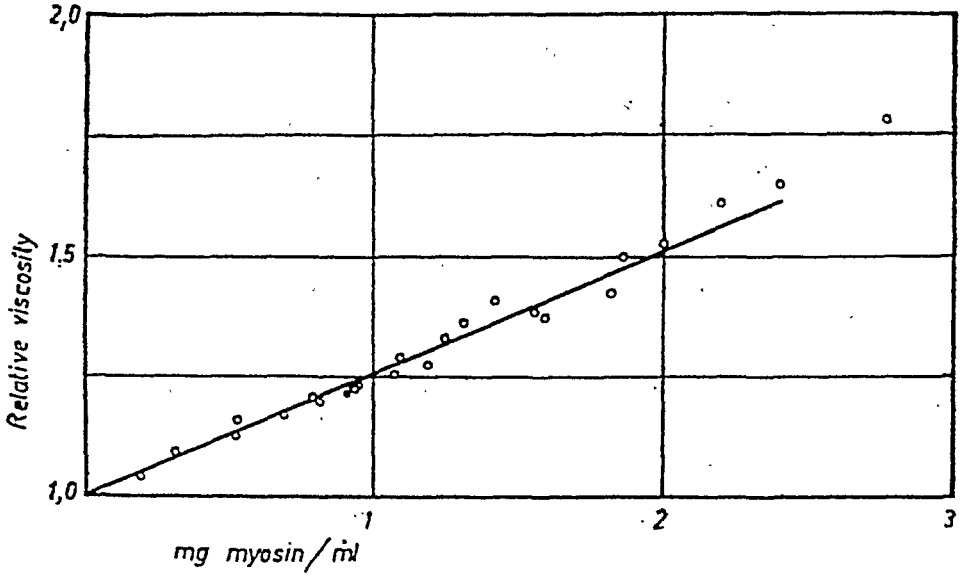


Fig. 5. Viscosity of recrystallised myosin in 0.6 *M* KCl at 0°.

DRF. Myosin, dissolved in water, has a strong DRF. This DRF is not due to the fibrous nature of the single myosin particles but to their association, for it is readily abolished by the addition of small amounts of salts (0.5 *M* KCl) or alkali (pH 8.3).

Viscosity. The viscosity of myosin has been studied by GUBA and STRAUB, whose results are summed up in Fig. 5. The dependence of viscosity on pressure was studied by the same authors. They found the viscosity of myosin, dissolved in 0.6 *M* KCl, normal, *i. e.* independent of pressure between 5—100 cm (water-pressure). This is in agreement with the straight nature of the curve (Fig. 5), which also indicates normal viscosity.

Later A. PITTONI extended these experiments down to 1 cm water-pressure. He found that freshly prepared myosin, dissolved in water, showed no viscosity-anomaly at all. On storage the vis-

cosity became anomalous. Addition of salt (0.5 *M* KCl) also made the viscosity, below 3 cm pressure, slightly anomalous.

The relatively high viscosity of myosin indicates that the myosin particle is not round but elongated. The low or lacking viscosity, anomaly, and the lack of DRF at alkaline reaction indicate, that this dimensional asymmetry can be but of a low degree. All the observations are in agreement with the assumption that myosin consists of moderately elongated particles which have a strong tendency of lateral association to rather long particles or swarms, the forces, holding these swarms together, being rather weak. This is in agreement also with the soft, unelastic nature of the myosin-gel. Myosin, in many respects, resembles soap, which consists of moderately elongated particles with a strong tendency of coaxial swarm formation and strong DRF. The dimensional asymmetry of soap is of the range 1 : 10. According to WEBER (75) the myosin micel has about the same degree of dimensional asymmetry, its length being at least 500 and its width no more than 45 Å.

If treated with strong urea the myosin micel of MW 10^6 g decomposes, according to WEBER and STÖVER, into particles of MW of about 10^5 g. On treatment with urea the viscosity of myosin drops to the level of the viscosity of globular proteins (F. GUBA, oral comm.). This suggests that the elongated myosin micel is built up of about 15 globular particles of MW 70,000, standing in a row.

Spreading. Actin-free myosin readily spreads on the surface of water.

Solubility. If the crystalline myosin precipitate is dialysed, it swells up to a glassy, soft, unelastic gel. If water is added gradually and the mass homogenised by means of strong stirring, a viscous solution is obtained which shows a slight opalescence and a strong DRF, both of which disappear readily on addition of alkali (pH 8.3). Myosin is thus soluble in water. It is insoluble in alcohol or acetone and is readily denatured by their action.

Reaction with salts. The most striking and unique property of myosin is that it is precipitated by small amounts of alkalisalts. If to the watery solution of myosin 0.006—0.04 *M* KCl is added, the protein is precipitated almost quantitatively. The maximum of precipitation lies about 0.02—0.025 *M* KCl, at which concentrations the precipitate has the smallest volume, but even as small concentrations as 0.001 *M* cause precipitation.

Higher salt-concentrations dissolve myosin. On addition of 0.1 *M* KCl to a watery myosin-solution, no more precipitate is obtained; at this KCl-concentration the myosin is still strongly associated, as indicated by the DRF, which begins to fade at 0.3 *M* and disappears at 0.4 *M* KCl. Dissolution is thus complete at 0.4 *M* KCl. Accordingly, a salt-free myosin-gel is readily dissolved by 0.4 *M* KCl, is poorly dissolved by 0.2—0.3 *M* KCl and remains undissolved below this concentration. Below 0.4 *M* KCl the myosin associates more and more as the salt-concentration is decreased. If a 3 % myosin-solution in KCl is gradually diluted, a strong turbidity appears as the salt-concentration drops below 0.4 *M*. On stirring, the fluid assumes a silky appearance. Between crossed Nicols a strong DRF appears. The turbidity, silky appearance and DRF increase on further dilution. At 0.05 *M* KCl the myosin becomes insoluble and the particles visible in the form of crystals. The crystalline suspension too has a silky appearance and a DRF which persists for a long time after stirring.

The action of KCl is not specific. Other alkali salts behave in a similar way. Their precipitating and dissolving action is compared in Tab. 1. $CaCl_2$ and $MgCl_2$ give a rather voluminous,

Table I.

	0.2	0.1	0.05	0.025	0.0125	0.006	0.003	0.0015	0.0008	0.0004
KCl	0	0	+	++	++	++	+	+	0	0
KF	+	+	+	++	++	++	++	++	+	0
KJ	0	0	+	++	++	+	0	0	0	0
LiCl	0	0	+	+	++	++	++	+	+	0
NaCl	0	+	+	++	++	++	+	+	0	0
$MgCl_2$	0	+	++	++	++	++	++	++	++	+
$CaCl_2$	0	+	++	++	++	++	++	++	++	+

0.5 ml of the salt solution was added to 2 ml of a 0.1 % salt free myosin solution. Upper line: the final molar concentration of the salt. 0 means solution + means turbidity or precipitation.

amorphous precipitate through the whole range without a well defined maximum. This makes it evident that the precipitation is rather the action of the cation than that of the anion. The

valency of the anion has less influence; KCl has about the same action as the equivalent K_2SO_4 and the precipitating action of K-phosphate is only slightly weaker.

If the action of KCl will be discussed with preference, this is not because the action of K is specific, but because K, standing in the middle of alkali-metals, is a good example. Its interest is increased by the fact that it is the main cation of the muscle fibre which contains but little Na and no other alkalimetals.

Reaction with glycogen. If a watery solution of myosin is mixed with a solution of glycogen, a precipitate is formed. If, for instance, equal parts of a 0.2 % myosin and 1 % glycogen are mixed, the protein is carried down quantitatively. The resulting glycogen-myosin complex is not soluble in dilute (0.1 *M*) KCl. If, however, a small amount (0.05 %) of ATP is added, the compound dissociates and the myosin and glycogen dissolve and the solution behaves now as a solution of free myosin. Soluble starch or dextrin give no precipitate with myosin under similar conditions.

Muscle contains a rather high concentration of ATP which is, as shown by ERDÖS (see part IV), in contact with myosin. In resting, normal muscle there is thus no myosin-glycogen complex. It is not impossible, however, that a decrease of ATP concentration will bring about the formation of the myosin-glycogen complex and starts off herewith the cycle of fermentation which restores the ATP concentration.

Reaction with ATP. ATP increases the dispersity of myosin. If added in small concentration (0.05 %) to a watery myosin-solution, the viscosity and DRF slightly decrease. In presence of ATP the precipitating action of KCl is weaker and potassium-phosphate causes no precipitation at all. The precipitated myosin may even be dissolved by the addition of ATP whereby the lost DRF returns.

Such a colloidal activity is not surprising in the case of a pentavalent anion, like ATP, which needs 4 K for its neutralisation. A slight solvatising action can already be observed with phosphate: potassium-phosphate precipitates myosin less strongly than the equivalent KCl. This solvatising action is still more pronounced with pyrophosphate.

The action of ATP shows that some sort of a reaction has taken place between myosin and ATP, that myosin has bound ATP. I. BANGA (unpublished) undertook to investigate the quantitative relations of this reaction. She proceeded as follows:

The crystalline brei of recrystallised myosin was dialysed over night. After having lost its salts it swelled up to a glassy mass. This glassy mass was squirted through a thin tube of 1 mm diameter into different ATP-solutions of 0°. The myosin formed a thin thread. The thread-suspension was gently stirred a 0° for 20 minutes, then rapidly centrifuged at the same temperature and treated with an excess of methylalcohol. The alcohol precipitated the myosin but not the ATP. The denatured myosin was strained through a cloth, pressed out, was washed on the cloth with more alcohol, then dried, weighed, suspended in 5 ml of 0.2 *M* acetate-buffer of pH 5.3 and placed into the boiling water-bath for ten minutes, and the fluid analysed. The ATP (acid-hydrolysable phosphate) was estimated according to LOHMANN-JENDRASSIK.

The myosin-gel contained about 4 % myosin. For the single tests about 200 mg of myosin were used which were squirted into 50 ml of a 0.1, 0.2, and 0.4 % ATP-solution. The ATP was employed in the form of its K salt.

The results of two such experiments are reproduced in Fig. 6. The abscissa shows the quantity of mg ATP per ml. The ordinate

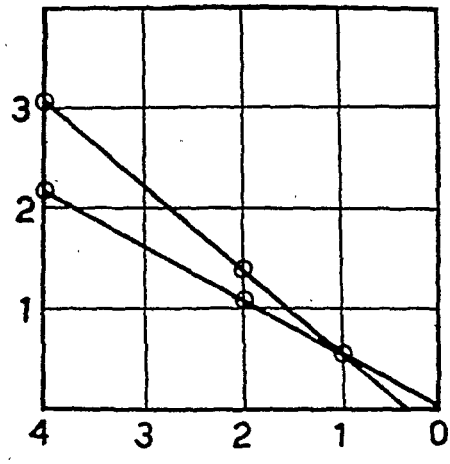


Fig. 6.

shows the number of moles of ATP bound by 70,000 g of myosin (or the number of ATP molecules bound by particles of myosin of MW 70,000 g). As will be seen, with increasing ATP-concentration more and more ATP is bound. The curves are straight and reach the abscissa about 0. ATP is thus not bound according to an exponential adsorption-isotherm. The curves rather suggest some sort of a chemical reaction between ATP and myosin. Muscle contains about 0.4 % of ATP, as calculated for 77 % of water. This corresponds to the highest value in Fig. 6. At this concentration 70,000 g myosin bind 2—3 moles of ATP.

Metal-myosinates.

As has been shown, myosin is precipitated by small amounts of alkali salts. The precipitation of a hydrophilic colloid by as small concentrations of KCl as 0.02 *M*, is a striking phenomenon. It is a unique reaction of myosin, the mechanism of which is of the greatest interest.

K. LAKI has shown (137, p. 82) that myosin, if precipitated by KCl, looses its charge and migrates no longer in the electric field or does so only very slowly. This shows that the cation of the salt is bound with preference and neutralises the negative charge of the protein, and that the precipitation of the protein is due to the loss of its charge.

If myosin is crystallised from dilute KCl, the crystals separated and treated with an excess of 0° alcohol, a powder of denatured myosin is obtained, which contains a considerable quantity of K but no Cl. Myosin thus binds the K leaving the Cl unbound; the precipitation is thus due to the formation of the electro-neutral and water-insoluble K-myosinate.

Denatured myosin does not bind K. If KCl is added to myosin, K is bound. If alcohol is added, the protein becomes denatured and releases the K again; the myosin is precipitated as free myosin, while the K enters with the Cl into equilibrium again. In order to precipitate myosin-K, we have to separate the K-proteinate from the Cl prior to the addition of alcohol. This can be done by removing the Cl-containing fluid. If the isolated K-myosinate is treated with alcohol, on denaturation it will be unable to release the K, no Cl being present to serve as its cation and no water being present to allow hydrolytic dissociation.

I. BANGA (unpublished) undertook to study quantitatively the formation metal-myosinate.

She proceeded as described for her ATP-experiments, the difference being that instead of an ATP-solution she used different salt-solutions. The threads were allowed to remain in the salt-solution for 40 minutes at room-temperature and for 20 minutes at 0°. For precipitation ethyl-alcohol instead of methyl was used. The K was estimated according to CRAMER and TISDALL, the Ca was precipitated as oxalate and titrated with permanganate. The Mg was precipitated as $Mg(NH_4)PO_4$, the P of which was estimated according to LOHMANN-JENDRASSIK. The K, Ca and Mg were employed in the form of their chlorides. The myosin-precipitate contained mostly no chlorine, only in the case of the highest saltconcentration was sometimes chlorine found in analysable quantities. In these cases the Cl was estimated according to VOLHARD and

its equivalent subtracted from the metal found in the precipitate. The solutions were neutral, myosin being used, crystallised at pH 7. No buffers could be employed, buffers being salts. On addition of salt to myosin there is a slight decrease of pH owing to the binding of metal.

K. The metal-adsorption was measured at varied salt-concentration and the quantity of metal plotted against the salt con-

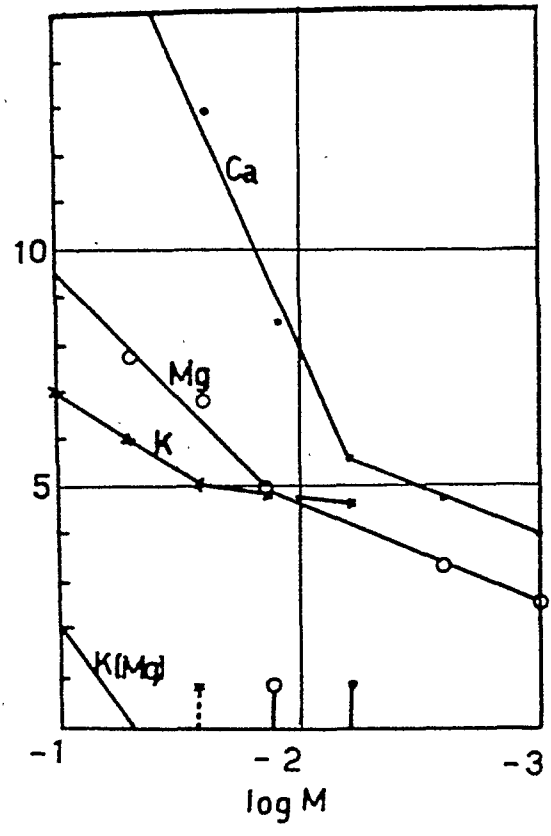


Fig. 7.

centration. The curves, thus obtained, are adsorption-curves of myosin and at the same time they are the dissociation-curves of the metal-myosinates. On the subsequent figure 7, taken from BANGA's unpublished paper, the abscissa shows the log. of the final *M* salt-concentration. The numbers of the ordinate denote the g-equivalents of metal bound by 17,600 g of myosin. The metal-adsorption will always be related to this quantity of myosin which will be taken as "the myosin unit". This will happen for the sake of convenience only and does not suggest that myosin actually reacts in such units.

In the Fig. 7 three experiments are reproduced in full line, one

with K, and one with Ca and Mg. The curves could not be extended further to either side because at a higher concentration the myosin dissolved, at a lower concentration it swelled.

All the three curves show a break in the middle and are composed of two straight parts. This shows that in the binding of metal by myosin two different adsorption-processes are involved, both corresponding to an exponential equation, similar to the adsorption-isoterm.

The meaning of the break is explained by the KCl-curve. The KCl concentration (0.025 *M*), which causes maximum precipitation of myosin, is marked with a vertical line and coincides with the break in the K-curve. Maximum-precipitation occurs when the myosin is completely discharged, is iso-electric. The right part of the curve corresponds to the K-adsorption of the negatively charged myosin: the left part of the curve describes the further K-adsorption of the electro-neutral K-myosinate, and the break occurs where the myosin has just taken up the 5 K ions necessary for its neutralisation. If less K^+ is taken up, myosin must have a negative charge; if more K^+ is taken up, the charge must be positive.

The physical properties of myosin depend on its charge, *viz.* K-saturation. With 5 K^+ adsorbed, myosin is isoelectric, is maximally precipitated and dehydrated. With 4.5 K^+ bound, the charge is negative and myosin begins to swell strongly; with two positive charges (7 K^+ adsorbed) the myosin begins to dissolve. If the curve is extrapolated it shows that myosin is completely disaggregated (0.4 *M* KCl) with 8—9 K^+ bound.

In the different experiments a variation was found in the shape and height of the K-curve. Often the left wing was less steep and consequently there was no break in the curve. Often the curve lay somewhat lower, and was flatter. The explanation of these variations was given by the experiments of F. Guba (unpublished) which showed that myosin is very sensitive and rapidly loses its K-binding capacity. First the K-binding capacity of the isoelectric myosin is damaged, the left wing becomes flat, and then the whole K-binding decreases. This is illustrated by the experiment reproduced in Fig. 8. In this experiment the myosin was crystallised only once, in order to have it possibly fresh. The protein was dialysed over night and tested the next morning (upper curve). Then it was stored for two days at 0° and tested again (lower curve). It will be seen that 48 hours

storage at 0° greatly changes the curve. Other experiments show that 24 hours storage already changes the shape of the curve to some extent.

After this experience was made, the preparation of the myosin with one recrystallisation, was always completed in one day, and dialysed over-night. In this way the protein was subjected to experiment 24 hours after killing the animal. With such preparations more constant results were obtained and the curves had regularly the shape given in Fig. 7. This curve, taken with recrystallised myosin, is somewhat flatter than the upper curve of Fig. 8, which relates to myosin crystallised only once. One may ask, whether the curve, *in vivo*, is not steeper still, if 24 hours make such a difference.

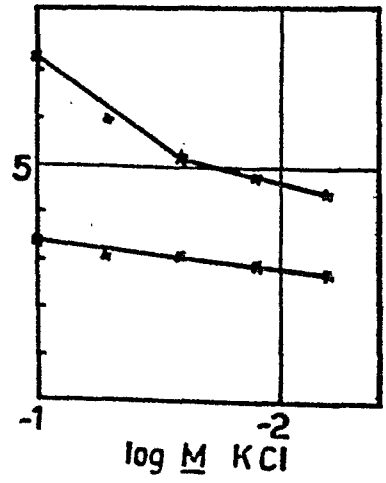


Fig. 8.

To these variations of the K-binding curves correspond variations in the colloidal reactions of myosin. Preparations with flatter curves react in a more sluggish way and need higher saltconcentration for the same effect. This explains the variations in the metal-concentrations, necessary to produce certain reactions in the author's colloidal experiments, which were not made with equally fresh myosin-preparations and need thus revision as regards the salt-concentrations at which certain reactions are produced.

Mg, Ca. The curves (Fig. 7) are similar to the K-curve with the difference that the left wing is steeper and the break occurs at a lower salt-concentration. The latter means that bivalent ions have a much greater affinity to myosin than K has and much smaller concentrations suffice to have 5 equivalents bound and to discharge myosin. This great affinity explains why 0.001 *M* Ca and Mg suffice to cause maximal precipitation. Mg stands between Ca and K. Accordingly it was found by BANGA that if Ca and Mg were present simultaneously, Ca was bound with preference and only traces of Mg were adsorbed, while both bivalent ions prevailed upon K.

It is interesting to compare the break of the three curves with the physiological concentrations. The Ca- and Mg-concentration

of muscle are marked on the curve by two vertical lines, while the K-concentration coincides with the ordinate (concentrations are calculated for the 77 % of water of muscle, DUBUISSON 17). It will be noted that these concentrations coincide, in the case of Ca and Mg, with the break of the curves. This means that the concentrations of these metals in muscle are such that they are unable to charge myosin positively. They are sufficient to produce isoelectric myosin, contrary to K, the concentration of which is high enough to charge myosin positively.

The steepness of the left wing of the three curves increases in the same order: K, Mg, Ca. It will also be seen that a higher positive charge is needed to dissolve Mg or Ca-myosinate than is needed for the dissolution of K-myosinate. The Ca-curve cuts the ordinate at about 20. More than 20 positive charges are needed pro unit myosin to bring the Ca-myosinate into solution.

All these reactions are reversible, as the curves also indicate. On diluting the salts solution metal is given off, on concentrating it, metal is taken up. Accordingly the metal can readily be removed by dialysis, the K the easiest.

Combined salt-effects. We may ask: what condition can we expect to find in muscle as judged by these curves? The muscle contains 0.105 *M* K, 0.012 *M* Mg and 0.006 *M* Ca, as calculated for 77 % of water (DUBUISSON 17). Provided these ions would be all free (which is not the case) we could expect Ca to be bound on the first place and produce isoelectric Ca-myosinate. If, however, the quantity of Ca and myosin are compared, it will be found that the quantity of Ca in muscle is insufficient to discharge myosin: about half as much Ca is present as would be needed for this reaction. Ca will thus take myosin half-way towards its isoelectric metal-saturation; the rest will be done by Mg.

If a K salt is added to such an isoelectric Ca-Mg-myosinate, the result depends on the concentration of the K. If, for instance, the K-concentration would only suffice to produce isoelectric myosin, no K is bound, for myosin is made isoelectric by the bivalent ions, and K is unable to compete with them. If, however, the KCl-concentration is increased to a level, which is able to charge the myosin positively, then K will be taken up. If 0.1 *M* KCl lends 7 charges to myosin, then, at this KCl-concentration 2 K^+ will be taken up, 5 charges having been lent by the bivalent ions. The K-adsorption-curve of isoelectric Ca- or

Mg-myosinate has been found to be steeper than the corresponding curve of myosin. Such a curve of isoelectric Mg-myosinate is reproduced in Fig. 7 with broken line.

If the salt-solution is diluted in such a system, then first the K will be given up. The Mg and Ca are held strongly and their quantity bound is dependent on the concentration only to a small extent.

pH. The H ion has a very great affinity to myosin and is capable of competing with the alkali-metals and alkali-earths. Accordingly, the pH will have a great influence on the metal-binding capacity of myosin. A K-curve, at varied pH and varied KCl concentration, is given in Fig. 9. The K-binding was measured at varied KCl concentration in a veronal-acetate buffer which contained, as metal, 0.01 *M* K. The K of the buffer was subtracted from the KCl; the abscissa shows the total K-concentration (F. GUBA). It will be seen that the metal-binding is very sensitive to pH, especially under pH 7. Decrease of pH by half a unit greatly decreases the metal-binding. According to this curve, the myosin, crystallised at pH 6.5 from a 0.025 *M* KCl is a myosin, containing 1 K per unit-weight. At pH 5.3 no K is bound at all.

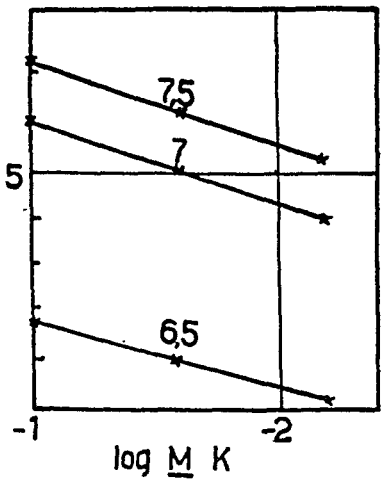


Fig. 9.

The effect of pH on the precipitation of myosin is shown in Tab. II. Here the buffer acted as the precipitating salt. The asterisks mean crystalline precipitate.

The table shows that the maximum of precipitation lies at pH 5.5 or below, but that the precipitation extends far into the alkaline-region. The maximum at low pH corresponds to the isoelectric point of free myosin, while at higher pH-s metal-myosinates of different composition are precipitated, which agrees with the conclusion of HOLLWEDE and WEBER. It can also be seen that crystallisation is limited to a small range. It is thus K-myosinate of a certain composition which is capable of crystallisation at its isoelectric point. According to the results presented it is the myosin, containing 1—5 K per unitweight, which is capable of crystallisation. The precipitation-maximum, found by different researchers at pH 6.5,

seems to be the isoelectric reaction of myosin, containing one K per unitweight.[1]

Table II.

$\frac{Na_2HPO_4}{KH_2PO_4}$	0.1	0.05	0.025	0.0125	0.006	0.003	0.0015	0.0008	pH
$^1/_0$	0	0	0	0	+	+	+	0	
$^{16}/_1$	0	0	0	++	++	+	+	0	8
$^8/_1$	0	0	0	++	++	+	+	0	7.7
$^4/_1$	0	0	+	++	++	+	+	0	7.3
$^2/_1$	0	0	+*	++	++	+	+	0	7
$^1/_1$	0	0	++**	+++	+++	+	+	0	6.7
$^1/_2$	0	+**	++**	++*	++	+	0	0	6.4
$^1/_4$	+	+	++	++	++	+	0	0	6.1
$^1/_8$	+	+	+	+	+	+	0	0	5.8
$^1/_{16}$	+	+	+	+	+	+	+	0	5.5
$^0/_1$	+	+	+	+	+	+	+	0	

Isomolar Na_2HPO_4 and KH_2PO_4 were mixed in different proportions (Col. 1). The final molar concentration of PO_4 is given in the upper line.

The metal-myosinate formation extends or shifts the isoelectric zone of myosin towards the alkaline side (as adsorption of Cl would probably extend it to the acid side). Other proteins show a similar shift or extension of the isoelectric zone in presence of salts as described by MICHAELIS and the author[2] for the case of casein. These effects are thus due to the binding of ions; the binding seems to depend on the affinity of the protein on one side, on the electronegativity of the ion (ERDÖ 120 and LAKI 121) on the other.

In spite of its striking appearance, the precipitation of myosin by alkali salts is not a specific reaction; it is a common reaction of proteins. It is in essence but an extension of the isoelectric zone, due to the unequal adsorption of ions. Myosin is specific

[1] This may explain why crystalline myosin-K is coloured by the basic methylenblue above, but not below pH 6.5 while the reverse is true for acid dyes.
[2] Biochem. Z. 103, 178, 1920.

only as far as its affinity to metals is especially high and thus the effect of the salt especially strong.

ATP. As has been shown, ATP unites with myosin to form a complex ATP-myosin with it. BANGA has studied the metal-binding capacity of this myosin-ATP-complex: she measured the metal-binding of myosin in presence of ATP.

She proceeded as in her previous experiments, using methylalcohol for the precipitation. The myosin was allowed to come into equilibrium with the salt for forty minutes at room-temperature, then cooled to 0°, the ATP introduced and the suspension centrifuged after 20 minutes stirring.

Her experiments showed that in presence of ATP the quantity of bound K is greatly increased by ATP, depending on the ATP-concentration. The two upper curves (broken line) of Fig. 10 show the K-binding in two different experiments in presence of 4 mg ATP per ml. The lowest curves show the number of ATP molecules bound by myosin-unit. ATP, at neutral reaction, needs 4 K for its neutralisation. So if ATP is bound by the myosin and is precipitated with it, it can be expected to bind this quantity of K. If the curves are corrected by subtracting this quantity of K, a normal K-binding curve is obtained (middle-curves).

The K-adsorption of myosin in any given solution of the K-salt of ATP, can be predicted with fair accuracy. The quantity of K bound will be equal to the sum of two magnitudes: 1, the K corresponding to the K-adsorption at the K-concentration of our solution. 2. the K, taken down by the ATP (mols of ATP-bound multiplied by 4). The binding of ATP can be read from Fig. 6.

ATP had less effect on the binding of Ca and Mg. If ATP is bound by myosin in presence of Ca or Mg and K, it takes down K with preference on precipitation by alcohol. Experiments of the ATP binding of Ca- or Mg-myosinate are rendered difficult by the insolubility of the Ca and Mg salts of myosin in alcohol.

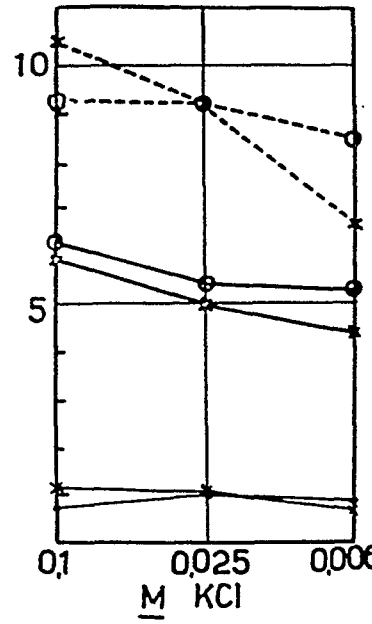


Fig. 10.

In several experiments the quantity of ATP, bound by myosin, increased with increasing KCl concentration above the isoelectric 0.025 *M* KCl. So, for instance, in one experiment the quantity of ATP bound was doubled by increasing the KCl-concentration from 0.025 to 0.1 *M*. This increased ATP-binding, on its turn, made the K-binding-curve very steep, one ATP taking down 4 K. It can easily be understood if a positively charged myosin binds more of the ATP-anion than a negative one. The effect, however, was not constant and in several experiments KCl had no effect on the ATP-binding capacity.

PART II.

Actin.

Actin is described by STRAUB in two papers (116, 117) and in one paper by BALENOVIĆ and STRAUB (117). In writing this chapter I will quote freely from these papers and will make only a few additions of my own.

Some properties and reactions of actin.

Actin, prepared by STRAUB's method, has the following composition:

C: 51.3 %, H: 8.6 %, N: 15.1 %, Ash: 1.1 %.

According to these data it is a protein. It contains no P.

The muscle extract, containing actin in high purity, is a limpid fluid. Its specific viscosity is very low: somewhat higher than that of hemoglobin and somewhat lower than that of serum albumin. It is also independent of the pressure, shows thus no anomaly (Fig. 11) and has no DRF. This proves that actin, in this state, is a globular protein.

If 0.1 *M* KCl is added to the neutralised extract, within a few minutes the fluid becomes slightly opalescent and develops a strong Tyndall effect. It becomes highly viscous and shows a splendid DRF, which persists for some time after stirring (Fig. 12). The sign of the DR is identical with that of myosin (positive). The viscosity is highly anomalous (Fig. 11). All this shows that actin, in this state, is a fibrous colloid.

A transformation has taken place under the influence of KCl: the globular protein has been transformed into a fibrous one. This transformation can be effected by any salt that does not destroy the protein. The fibrous form reverts to the globular form if dialysed at an alkaline reaction: the transformation is reversible.

Actin can thus exist in two forms: as the globular G-actin ("G" stands for globular) and as the fibrous F-actin ("F" stands for fibrous). This transformation will be called here the G—F transformation. STRAUB had called this process the "activation" of actin, F being the "active", G the "inactive" actin. By "activity" he meant the ability of actin to form a highly viscous actomyosin. This is done by F-actin only and not by G-actin.

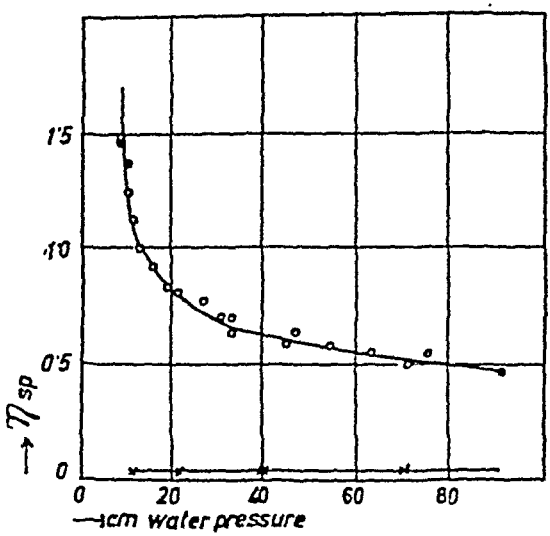


Fig. 11. Viscosity of inactive and active actin at different pressures.
o—o 2 mg/ml active actin in 0.2 *M* KCl.
x—x 3 mg/ml inactive actin in dist. water at 0°.

This property of actin, that it is capable of existing in two forms, in globular and fibrous form, which can be reversibly transformed into each other, is striking and unique.

As to the nature of the G—F transformation of actin, two possibilities present themselves. Either the globules are polymerised in such a way that they form, like a string of beads, a linear particle, or else the globules are unwound to a thread. Observations, made up till the present, are more in harmony with the polymerisation (increase in opalescence, development of Tyndall effect), so I will employ this explanation.

In any case the linear actin particles is formed of globular particles. The same is probable for myosin, as shown before. A. PITTONI (unpublished) has obtained results with fibrinogen which suggest that this linear colloid has an analogous structure; it may be asked whether the globular form is not the basic form of protein and whether not all fibrous proteins are built up of globular particles.

Viscosity and Thixotropy of F-actin. F-actin is thixotropic. If a 0.4 % F-actin solution is allowed to stand it gelatinises. Gentle shaking breaks up the gel into a fluid. Thus the cohesive forces, which cause thixotropy, are rather weak and are easily broken. Probably this explains why there has been some divergence of opinion about the thixotropy of actin. STRAUB, in his first paper, states that actin is thixotropic. In his second paper he takes this

Fig. 12. Double refraction of actin solutions.
Photographs taken through crossed nicols. Left: inactive actin.
Right: the same solution after activation.

statement back. GUBA, who has devoted since then a great deal of work to actin, finds this colloid distinctly thixotropic. The explanation of these discrepancies may be that small forces, like gentle motion, suffice to break up the thixotropic structure. So subsequent readings in the viscosimeter may give the same values, the structure having been broken up completely by the streaming at the first reading, while other thixotropic colloids break up gradually and give decreasing values under the same condition in the subsequent readings.

Thixotropy makes it impossible to state exactly the specific viscosity of actin. All the same, under the usual conditions of viscosimetry, that will say, if we employ not too low pressures or not too concentrated solutions and not too narrow capillaries, the influence of thixotropy will be small, no more than 10 per cent.

The thixotropy indicates that the actin particle has strong cohesive forces which find their explanation in the function of actin which probably forms long and strong threads in the muscle fibril.

Viscosity. In Fig. 13 the dependence of the specific viscosity of F-actin on concentration is given (taken from an unpublished paper of GUBA). The curve shows that F-actin has about the same specific viscosity as myosin. While this viscosity is much higher than that of globular proteins, it is much lower than that of highly polymer substances, like rubber or cellulose.

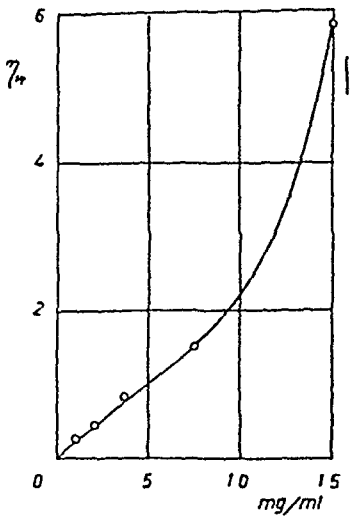


Fig. 13.

If the viscosity is taken as an index of the length of the particles, then it indicates that the particles of F-actin are of the same length as those of myosin. Neither the strong DRF nor the high degree of viscosity anomaly are explained by the length of the particles. The high degree of anomaly is, in all probability, due to the thixotropy, the intense DRF to swarm formation, with coaxial association of particles.

While the DRF of myosin is very sensitive to pH and salt concentration and is easily abolished by their increase, the DRF of actin is not sensitive. The DRF of diluted actin is abolished by higher alkalinity or acidity, but the loss is irreversible. Moreover, while the DRF of myosin ceases as soon as the flow has stopped, a stronger F-actin solution shows permanent DR. This shows that the coaxially arranged and associated particles are held together by stronger forces than in the case of myosin — probably the same forces make actin thixotropic.

That actin is not a really highly polymer substance, comparable to rubber or cellulose, is also shown by the fact that if dried at neutral reaction, it dries down to a hard, thin sheet, and does not form a resinous mass like highly polymer substances.

Reaction with salts. Any salt, in high concentration, will precipitate actin. KCl, for instance, will precipitate it at 2 *M*. Ca and Mg, however, precipitate the protein in high dilution: 0.002 *M* $CaCl_2$ or $MgCl_2$ will suffice to bring it down. The precipitation

sets slowly and entails, at room temperature, partial denaturation. Mn and Sr have a similar effect.

The Ca and Mg precipitation at 0° can also be used for the purification of actin. Precipitation with Ca has its maximum at 0.1 *N* concentration. Higher concentrations, like 0.6 *N* cause no more precipitation. The precipitated actin can be centrifuged out and liberated from the bulk of Ca by means of dialysis at 0°. The small quantity of remaining Ca can be precipitated by oxalate.

There is a distinct antagonism between Ca and K. KCl, if added in sufficient quantity, will not only prevent precipitation by $CaCl_2$, but will even dissolve the Ca precipitate, especially if added immediately after the precipitate was formed. The redissolved actin will be more a suspension than a solution, but all the same it will be active towards myosin. After the precipitate has been standing for some time, the redissolution will be more incomplete and there will be a loss of activity. For complete dissolution 40—50 times as much K have to be added as Ca. Na has a similar effect to K. This relation of Ca to K and Na corresponds to the values of the well known physiological antagonism of these ions.

NaJ has a special action. In low concentration (0.1 *M*), it "activates" the actin, like any other salt, say KCl, 0.5 *M* NaJ does not activate at all. If this concentration of NaJ is added to F-actin, within 30 minutes, at 0°—22°, the DRF disappears and the viscosity falls to very low values which correspond to a globular protein. NaJ thus disintegrates the F-actin into G-actin. The effect is irreversible. If the salt is removed by dialysis, the actin remains "inactive" and cannot be activated again. Bromides have a similar but much weaker effect.

pH. Actin is precipitated by acids with a maximum at pH 4.7. The precipitate can be redissolved at pH 7. Salts, if present during precipitation, denature the protein.

G-actin is precipitated under these conditions as a very dense precipitate which settles very quickly and to a small volume. It will be redissolved partly activited to F-actin. If pH 4.7 buffer is added to the F-actin, this does not precipitate but forms a transparent mass. Shaking breaks up the structure and the actin flocculates forming a bulky precipitate. Actin, dissolved in acid, is very sensitive towards anions: chlorides cause immediate precipitation and denaturation, whereas phosphates do not precipitate.

The viscosity of F-actin greatly depends on the pH. The pH dependence of viscosity is given by Fig. 14 taken from the unpublished paper of GUBA.

Stability. Actin is easily destroyed by heat over 50°. It is stable in a narrow pH zone between pH 7—7.5 but is destroyed even at 0° by more acid and alkaline reactions. Dialysis at pH 7 does not diminish its activity. It can be precipitated by cold alcohol but it is completely destroyed if treated with cold acetone. (This is the more interesting as it is very resistant to acetone when still in the muscle tissue.) Actin, precipitated at pH 4.7 can be treated with acetone but is readily denatured and rendered insoluble by drying on air. At neutral reaction actin can be dried in the *vacuum* desiccator without being denatured, and can be redissolved in active form.

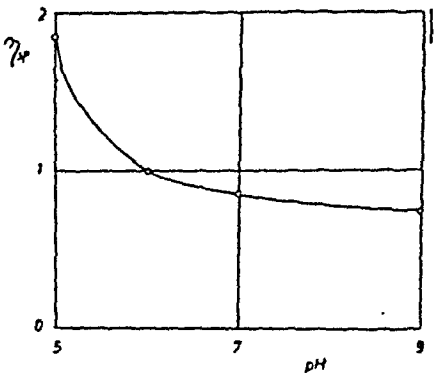


Fig. 14.

Solubility. Actin is soluble in water but not soluble in anhydrous solvents like acetone, alcohol or pyridine. A great excess of alcohol has to be added to its neutral or alkaline solution to precipitate it.

The G—F transformation (activation).

The end-point of activation is independent of the nature of the salt used. As non-dissociating substances have no effect, the activation must be ascribed to *ions*. The concentration of salt needed for activation is inversely proportional to the charge of the positive ion.

About 10 times more KCl is needed, than $CaCl_2$, to produce the same effect. Of the alkali chlorides, the velocity of activation increases in the order: Rb, K, Na, Li. If the haloids (used as Na salts) are compared, the velocity of activation increases in the order: J, Br, Cl, F. Thus NaF activates very fast, KJ very slowly. The effect of the charge of the anion is opposite to the effect of the charge of the cation, e. g. sulfates activate much slower than chlorides. The hydrogen ion is a more potent activator than

any other ion: there is instantaneous activation at a pH, lower than 6.

The velocity of activation depends upon the concentration of the salt. If a low concentration of salt is used, the time curve of activation can be studied in the viscosimeter. It is found that after a rapid rise the activation is considerably slowed down and the end point is reached very slowly. This fact points to the multimolecular nature of the reaction (Fig. 15).

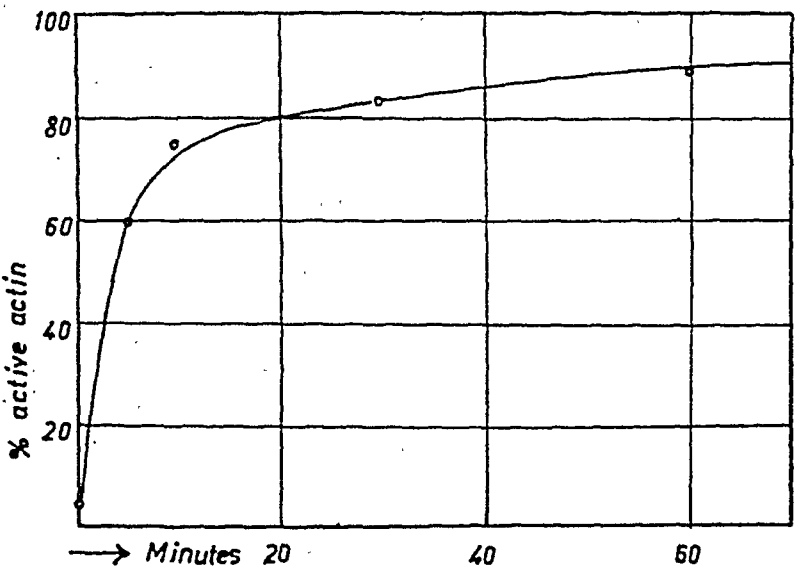


Fig. 15. Activation time curve in 0.02 *M* NaF at pH 6.5 and 35.5°.

The effect of temperature is shown in Tab. III. The velocity of activation rises rapidly with increasing temperature.

Table III.

Temperature	% active actin found
0	4
12	70
20	82
30	100
35.5	100

Effect of salt concentration. (Fig. 16.) It is almost impossible to get a true picture of the effect of salt concentration because the end-point and the velocity of activation both vary with the salt-

concentration. The end-point is reached very slowly with smaller concentrations of salt.

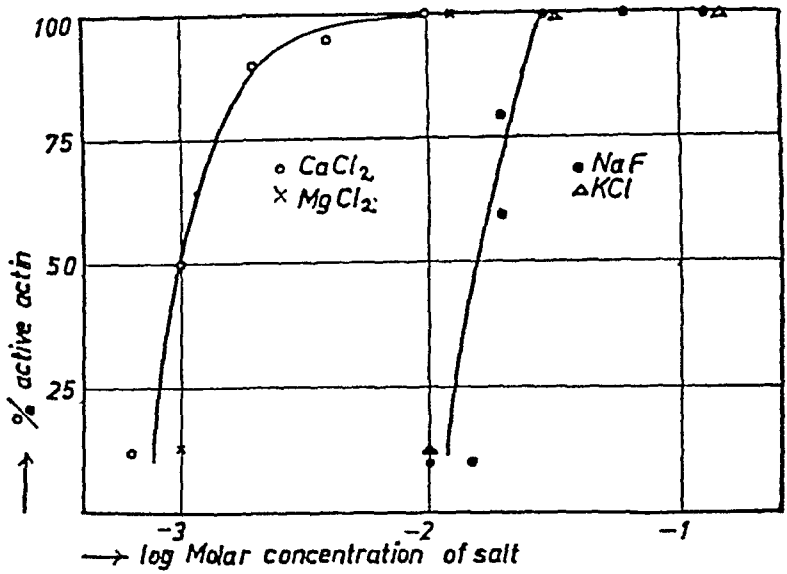


Fig. 16. Effect of concentration of salt on the activation of actin. 2.5 mg/ml actin incubated at 35.5 with salt of the concentration indicated on the abscissa. pH 7.4. The % active actin content was determined after 60 minutes' incubation.

The pH-dependence is shown in Tab. IV.

Table IV.

Actin incubated for 15 min. at 22° at pH	% active actin found
7.0	6
6.0	20
5.7	75
5.3	100

Reversibility. If the salt, which caused activation, is removed, the actin remains active. Inactive actin is, however, formed during dialysis if the actin solution is first made alkaline. An actin solution, which contained 70 % of the actin in the active form, was mixed with 1/10 of its volume of 0.1 *M* borate buffer of pH 10 and dialysed against CO_2-free distilled water for 24 hours. The dialysed solution contained only 2 % of the actin in its active form, the rest being inactive actin, which could be activated by the addition of salt. The total actin content decreased by 30 % during dialysis.

Effect of freezing. If G-actin solution is frozen, on thawing the actin will be found activated partly to F-actin. This may be explained by the effect of freezing on the actin- and salt-concentration, which are greatly increased by the crystallisation of water, which both enhance activation. This method of activation is very valuable if an F-actin solution is needed, poor in salt.

Mg and Ca. STRAUB found that on addition of 0.1 *M* KCl at 0° G-actin was readily transformed into F-actin. This was true for a fresh actin preparation. If, however, the actin solution was stored, the rate of transformation rapidly decreased and after a few hours there was no activation at all. This was not due to the destruction of the actin, since, at room temperature and at a higher actin concentration, the protein could still be activated to a full extent.

The low rate of the transformation of stored actin at 0° made it possible to follow this process in the viscosimeter, the G—F transformation being accompanied by a strong rise of viscosity.

In Fig. 17, taken from the last unpublished paper of STRAUB, this influence of the storage on the rate of the G—F transformation is shown. It will be seen that one hour of storage suffices to slow down the process to a very great extent.

Curve 2 of Fig. 18 shows, that the viscosity of a stored actin rose no more in the presence of 0.1 *M* NaCl, there was thus no G—F transformation at all.

STRAUB has found (unpublished) that addition of a small amount of watery muscle extract greatly increased the rate of transformation. This effect was due to the presence of two factors, the one thermolabile, the other thermostable. The labile factor was found to be myosin.

Myosin, in quantities of 0.5—1.0 mg, given to 8 mg of actin, increases the rate of the G—F transformation (0.1 *M* NaCl) 5—10 fold. This is true if the actin is still capable of some activation. If it has been stored too long and is not activated by NaCl any more, addition of myosin will not change this result. Thus myosin only speeds up the salt-activation but is unable to replace it. While myosin speeds up activation in presence of low salt concentrations, in presence of high salt concentrations it has the opposite effect. These higher salt concentrations (*e. g.* 0.6 *M* KCl), which readily activate actin in absence of myosin, are inactive in its presence.

Actin in muscle.

According to BALENOVIC and STRAUB 1 g of rabbit's muscle contains 25—30 mg of actin, representing about 12—15 % of the total protein. It is evident that the insoluble "stroma", which makes about 15—20 % of the total protein of muscle, must chiefly consist of actin. If blood vessels and connective tissues are discounted, no room is left for any other protein to play a significant quantitative rôle in the composition of muscle. It seems thus to be incorrect to say, that actin "is fixed to the insoluble structure". We rather meet facts by saying that actin forms the insoluble structure itself.

PART III.

Actomyosin.

General remarks.

If a dilute solution of myosin and F-actin are mixed, the sudden rise of viscosity indicates the formation of a new, highly viscous substance: F-actomyosin, a compound of F-actin and myosin. The formation of actomyosin is reversible and the complex readily dissociates under different conditions into its components, actin and myosin.

The different reactions of actomyosin are mostly the reactions of one of its components. Actin being a rather inactive substance, most reactions of actomyosin are the reactions of its myosin moiety. In many cases this actin-linked myosin reacts in the same way as free actin does. So, for instance, if actomyosin is precipitated by alkali-salts and binds alkalimetals much in the same way as myosin does, this is evidently due to the reaction of myosin, which was not changed by actomyosin-formation, since actin is not precipitated by alkali metals at all.

In other instances, however, the reactions of myosin are altered by the actomyosin-formation. To give an example: Mg-myosinate is enzymatically inactive and is unable to split ATP. By its being linked to actin this Mg-myosinate becomes enzymatically active. While Mg inhibits free actin, it activates actin-bound myosin. Another example: free myosin is unable to split isomerised ADP, while actomyosin readily splits it. Since actin has no enzymic activity whatever, it is evident that this ADP-splitting is a reaction of the myosin induced by the actin.

Both F- and G-actin form actomyosin. The properties of the corresponding actomyosins are widely different and will be dealt with in special chapters.

Actomyosin is not a substance, defined by stoichiometric relations. According to the relative quantity of actin and myosin, different actomyosins are formed, which have their own physical constants, behave thus as individual substances. So, for instance, a 2 % F-actomyosin, containing no more than 2 parts of F-actin to 98 parts of myosin, will have its own characteristics.

The maximum "activity" is shown by an actomyosin containing two parts of actin to five parts of myosin (STRAUB 116, 127). According to BALENOVIC and STRAUB this is the relation of actin and myosin in muscle and if not stated otherwise, my text will relate to such actomyosin, prepared from pure actin and recrystallised myosin.

The most striking reaction of F-actomyosin is its contraction precipitation and dehydration under influence of salt and ATP. Salts, in absence of ATP, have the same action but only to a small extent. These reactions can be studied in gels or suspensions. If gels are used they are applied with advantage in the form of thin threads, prepared according to WEBER; a special chapter will be devoted to them. If suspensions are used, we may provoke precipitation or contraction. If our suspension is discontinuous, consisting of flocculi the contraction of the single flocculus will cause precipitation. If the suspension is more homogeneous and corresponds rather to a very dilute gel, then, under influence of salt and ATP, it will contract to a small plug, leaving a clear fluid behind (Fig. 19). This contraction is perhaps less striking than that seen in threads (Fig. 1) but diffusion will not limit reactions and one may see in a series of tubes dissolution and precipitation side by side.

Fig. 19.

Like myosin, actomyosin also is dissolved by higher concentrations of alkali salts. By an "actomyosin solution" I will mean, if not stated otherwise, actomyosin, dissolved in 0.6 *M* KCl.

F-actomyosin.

F-actomyosin is a hydrophilic colloid. At low concentrations it forms stable and transparent, highly viscous suspensions. At higher concentrations it forms a transparent, elastic gel.

The viscosity of actomyosin solutions is not only high but is also strongly anomalous. This is revealed by measuring the viscosity at varied pressure or concentration (Fig. 49, top-curve). Actomyosin solutions show also a splendid DRF, which is stronger than the DRF of its components (in 0.6 *M* KCl myosin has no DRF at all).

Actomyosin has thus all the characteristics of a typical highly polymer fibrous colloid. Since the high viscosity and the DRF are not effected by slight variations of pH or salt concentration, they cannot be due to a superficial association, like swarm-formation, but must be due to the high dimensional asymmetry of the actomyosin particles. This is rather remarkable because actomyosin is formed of two colloids none of which has such a high degree of asymmetry.

While the viscosity of actomyosin, extracted as such from muscle (myosin B) remains unchanged for a long time, the initial high viscosity of actomyosin solution, prepared from pure actin and myosin, rapidly drops to low values on storage even at 0°. The persisting strong DRF indicates that the drop of viscosity was not due to the disintegration of the F-actin but to the dissociation into actin and myosin. F. Guba, who made these observations (unpublished), found, that the viscosity could be stabilised by the addition of small amounts of muscle extract. The analysis showed that it was the Mg which was responsible for this effect which could be duplicated by the addition of small amounts (0.001 *M*) of $MgCl_2$.

According to the observations of F. Guba (unpublished), actomyosin is slightly but distinctly thixotropic, which is evidently due to the thixotropic nature of actin. The thixotropy is not high and the drop of viscosity, observed on repeated readings, hardly exceeds 10 % of the total viscosity. All the same, this thixotropy makes it impossible to state numerically the viscosity of actomyosin. Working, however, under identical conditions, with the same viscosimeter at the same pressure, the viscosity of different actomyosin preparations may be compared with satisfactory reproducibility. It was found to be the most convenient to compare the first readings.

Reaction with salts. If a watery myosin- and actin-solution are mixed, the sudden rise of viscosity indicates the formation of actomyosin. In fig. 20 the two columns on the left side show the specific viscosity of a 0.175 % myosin and a 0.07 % actin solution.

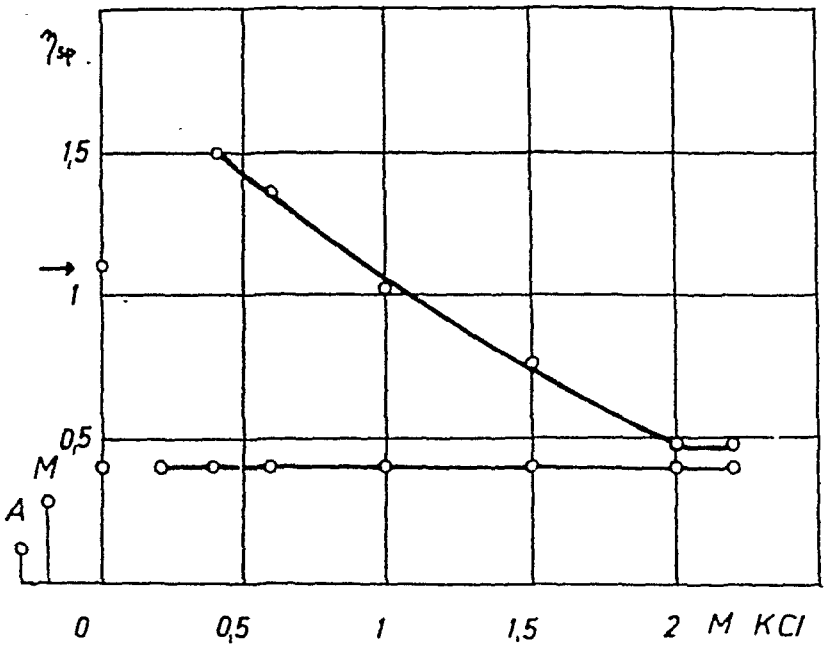


Fig. 20. Specific viscosity of actomyosin at varied KCl-concentration in presence (lower curve) and absence of ATP (upper curve). 170 % active actomyosin, containing 1.75 mg myosin and 0.7 mg actin per ml. 0.01 *M* veronal-acetate buffer of pH 7 in all samples. 0.015 % ATP. The vertical lines on the left indicate the specific viscosity of the corresponding myosin and actin solutions. The viscosities of salt-free actomyosin with and without ATP (arrow) are marked on the ordinate.

The viscosity of the corresponding actomyosin is marked on the ordinate by an arrow.

Though insoluble in water, actomyosin is very hydrophilic. At higher dilution it forms transparent, stable suspensions while at higher concentration it forms a transparent, elastic gel.

Similary to myosin, actomyosin also is precipitated by small amounts of alkali-salts. Accordingly, if KCl is added to a salt-free actomyosin-gel, it becomes turbid and shrinks to some extent, while if a salt-containing gel is washed salt-free, it clears up and swells greatly. Very small salt-concentrations, like 0.001 *M* KCl prevent this swelling.

The maximum of precipitation lies in the case of KCl, at 0.05 *M*, but even this maximally precipitated actomyosin is rather hydrophilic. Its flocculi are loose and, if centrifuged, enclose about 98 % of water. Also the actomyosin gel, at this salt concentration, is a hydrophilic colloid, which always encloses a very great volume of water. The author never succeeded in preparing an actomyosin which contained less than 95 % of water.

If the precipitation of myosin and actomyosin are compared at varied KCl concentration (Tab. V), it will be found to be identical at low salt concentration. The maximum of precipitation, however, is shifted in the case of actomyosin towards the higher salt concentration. At 0.05 *M* KCl, where the maximum

Table V.

M KCl	0,4	0,3	0,2	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	0
Acto-myosin	0	+	++	++	+++	+++	++	++	+	+	0	0
Myosin	0	0	0	0	+	++	++	++	+	+	0	0

Upper line: molar concentration of KCl. Crosses mean precipitation. 0.1 % solution of myosin and 0.1 % suspension of a 25 % actomyosin.

lies, myosin is positively charged and actomyosin is a compound of a positive and negative colloid, while the whole particle, formed from a cation and an anion, is isoelectric. While the isoelectric KCl-concentration of myosin is 0.025 *M*, that of actomyosin is 0.05 M. At a lower KCl-concentration the actomyosin is charged negatively, at a higher concentration it is charged positively.

Actomyosin is kept in solution by 0.4 *M* KCl. At this salt-concentration it has a high viscosity which gradually drops as the molarity of the salt increases. This is shown in the upper curve of Fig. 20 (F. Guba, unpublished). The minimum is reached at 2 *M* KCl where the viscosity corresponds roughly to the additive value of the viscosities of the actin and myosin present. This indicates that the actomyosin has dissociated completely into its components.

As is mostly the case with hydrophilic colloids, the solubility has no sharp limits and depends on conditions. 0.4 *M* KCl keeps actomyosin in solution, but an actomyosin gel is not dissolved even by 1.0 *M* KCl, especially if it has been standing for some time. In an actomyosin gel or precipitate cohesive links are formed which inhibit dissolution. The solubility of an actomyosin precipitate may decrease appreciably within seconds after its formation.

Like myosin, actomyosin also is strongly precipitated and made to shrink by bivalent cations. Even 0.0006 *M* $MgCl_2$ causes strong precipitation.

Evidently, all these colloidal reactions of actomyosin depend

on the charge, viz. the metal-saturation of the myosin. BANGA (unpublished) extended her studies on metal adsorption to actomyosin. She proceeded as follows:

2 *M* KCl was added to recrystallised myosin up to a final concentration of 0.5 *M*. To this myosin-solution was added a strong solution of actin, dissolved in 0.5 *M* KCl and purified by isoelectric precipitation. The solution contained enough actin to make maximally active actomyosin. The actomyosin solution thus obtained was squirted slowly through a thin tube into an amout of distilled water which sufficed to bring down the total KCl-concentration to 0.001 *M*. Here the actomyosin gelatinised in the form of a thin thread. The thread suspension was gently agitated for an hour, whereby the KCl was washed out and the threads swelled. The washed threads were suspended in the different salt solutions and then treated, as described for myosin.

These experiments showed that there was no great difference between the K-adsorption of myosin and actomyosin, if calculated for the myosin present. Actomyosin adsorbed more Ca and Mg, especially at a low salt-concentration, where actomyosin adsorbed nearly twice as much metal as myosin. The break of the curve was also less sharp. The difference is due, in all probability, to the binding of metal by actin, which is also precipitated by Ca and Mg but not by K.

The maximum of the precipitation of actomyosin lies between 0.025 and 0.05 *M* KCl where the myosin has bound 5 K. With one K less actomyosin swells strongly. With 8—9 K it is soluble and begins to dissociate (0.4 *M* KCl, extrapolated).

With about 11 K adsorbed its dissociation is complete (2 *M*). It is impossible to state yet the exact relation between charge and physical state because the K-adsorption-curves vary to some extent from preparation to preparation.

Actin unites with negatively as well as with positively charged myosin, provided the charge does not exceed a certain value. With 9 K the binding between actin and myosin becomes rather loose, as indicated by the incipient dissociation. With 11 K the myosin forms no actomyosin at all.

Reaction with ATP. Small amounts of ATP change the reaction of actomyosin to KCl in a striking way; the ATP-complex of actomyosin, actomyosin-ATP, reacts differently to KCl than actomyosin does. Actomyosin and actomyosin-ATP are two different substances. The lower curve in Fig. 20 shows the behavior of actomyosin-ATP (actomyosin in presence of 0.015 % ATP). As the curve shows the zone of precipitation has become narrower

and outside this zone, in absence of salt or above 0.2 *M* KCl, dissociation is complete throughout and the low viscosity corresponds to the additive value of myosin and actin. Actomyosin-ATP is thus either precipitated or dissociated; dissolved actomyosin-ATP does not exist and even the precipitated actomyosin-ATP is capable of existence only in a narrower zone of KCl-concentrations.

Not only the extent, also the nature of the precipitation has greatly changed. While the precipitated actomyosin is loose, floccular and rather hydrophilic, the precipitated actomyosin-ATP precipitate is granular and settles quickly to a small volume.

The difference between the precipitated actomyosin and its ATP-complex can be observed if ATP is added to a salt-precipitated actomyosin. On addition of ATP the loose, flocculent precipitate becomes granular and its volume decreases. The change is still more striking if the ATP is added to a piece of actomyosin gel, say an actomyosin-thread, suspended in 0.05 *M* KCl. This gel consists of salt-precipitated actomyosin and contains a great amount—mostly 98 % or more—water. On addition of ATP the thread rapidly shrinks to a small volume (Fig. 1 a and b) and presses out practically all water. The shrinking is so fast that it makes the impression of active contraction. The same effect can be observed on a more concentrated but homogeneous actomyosin-suspension which, on addition of ATP, contracts to a small plug, leaving a clear fluid behind (Fig. 19). Contraction and precipitation are thus identical phenomena. Contraction is but the precipitation of a continuous gel. If a discontinuous suspension contracts, it precipitates; if a continuous gel precipitates, it contracts. The change is due, as will be shown later, to the same basic reaction: to the shortening and complete dehydration of the actomyosin-ATP particle. The actomyosin-ATP particle is shorter and is less hydrated than the corresponding actomyosin particle. Thus the entering of the ATP molecule into the actomyosin-complex entails the contraction and dehydration of this latter. Similarly, if the distended and hydrated myosin-ATP particle unites with actin to form actomyosin-ATP, this entails contraction and dehydration.

The KCl-maximum of precipitation or contraction of actomyosin-ATP lies at 0.05 *M*, the same KCl-concentration at which actomyosin is maximally precipitated. Actomyosin-ATP is thus stable only in a narrow range of KCl-concentrations about its

isoelectric reaction, where the whole particle has no charge, actin is negative, myosin weakly positive. Outside this zone the ATP-complex of actomyosin dissociates, contrary to actomyosin which is stable in a much wider range of KCl concentrations and is stable even in the entire absence of KCl.

The formation and dissociation of actomyosin-ATP, is thus a matter of charge and metal-fixation. BANGA has studied the K-binding of actomyosin-ATP, that will say, measured the K-absorption curves of actomyosin in presence of ATP. She found that ATP (0.1 %) made no difference to the metal-fixation of actomyosin. More exactly: there was no other difference between the metal-fixation of actomyosin and acto-myosin-ATP than there was between myosin and myosin-ATP. Also the ATP-binding of myosin and actomyosin were equal (calculated for the myosin present). It follows thus that if the contracted actomyosin-ATP is formed from actomyosin and ATP or of myosin-ATP and actin, only the hydrate-water is given off, and not the metal. Contraction entails thus no loss of metal, only loss of hydrate-water.

At 0.05 *M* KCl, where actomyosin-ATP is the most stable, the most contracted and dehydrated, its myosin binds 6 K, is thus slightly positive and the isoelectric actomyosin is the compound of a positive and a negative colloid. How far this charge of myosin can be increased or decreased without causing dissociation of the complex, *i. e.* how wide is the stability-range of the complex at varied KCl-concentration, depends on the ATP content of this complex, which, on its turn, depends on the ATP-concentration of the solution. If there is no ATP at all, the zone is very wide; if there is little ATP present, the zone is narrower, and if the ATP-concentration is high, the zone is very narrow. The more ATP the complex contains, the stronger its tendency to dissociate and the less change in charge will suffice to cause its dissociation.[1] This is shown by the experiment reproduced in Tab. VI. It can be seen that the higher the ATP concentration, the narrower the zone of precipitation (contraction). At the physiological level of 4 mg ATP per ml the zone of precipitation is limited to a narrow range around 0.05 *M* KCl. While in absence of ATP the myosin unites with actin from 0—2 *M* KCl, from no K^+ to 11 K^+ bound, in presence of 0.015 %

[1] ATP caused dissociation also of the myosin-glycogen-complex. The ATP changes thus the general properties of myosin and its action is not directed towards the actin-myosin bond in a specific way.

ATP the zone is limited from to 0.003—0.2 *M* KCl which corresponds to 4—8 K^+ bound. At the physiological ATP-concentration the loss of a half, or the gain of 2 K^+ will suffice to cause dissociation, and contraction is limited to the narrow zone where myosin has a weak positive charge.[1] If actin and myosin-ATP

Table VI.

M KCl	% ATP						
	0,4	0,2	0,1	0,05	0,025	0,0125	0
0.1	! ! !	! ! !	! ! !	! ! !	! ! !	! ! !	! !
0.3	! ! !	! ! !	! !	! !	!	0	!
0.2	! !	!	xx	xx	xx	x	+
0.05	xxx	xxx	xxx	xxx	xx	xx	—
0.0125	!	!	0	0	x	x	—
0.003	! !	! !	!	!	!	x	—
0.0008	! ! !	! ! !	! ! !	! !	!	0	0

0.1 % suspension of 25 % actomyosin.
+ = precipitation, x = contraction, ! = dissolution, 0 = no change.

unite thus to form the contracted actomyosin-ATP, this contraction can be described, in terms of colloidal-chemistry, as the precipitation of a positive colloid by a negative one.[2] If the myosin-ATP has a different charge it will not unite with actin or if the charge of the myosin-ATP is changed within the actomyosin-ATP-complex (by changing the KCl-concentration), the complex dissociates into actin and myosin-ATP again. There is thus no real difference between contraction (association) and dissociation (relaxation), both are reactions of the same two colloids depending on the charge of their particles. The same reactions are given by actin and myosin also in absence of ATP. In presence of 0.05 *M* KCl actin and myosin precipitate, dehydrate and contract also to some extent but all these changes are very much weaker in

[1] The numeric statement of charge relates only to the myosin-preparations used in these experiments. It is probable that the K-dissociation-curve of myosin in vivo is somewhat different and accordingly also the numbers will be different.
[2] Actin is precipitated in a similar way by other positive colloids, like protamin.

absence of ATP and for dissociation we need much more extreme charges. What the mechanism of the reaction is by which ATP intensifies these reactions to such an extent, remains to be shown. Probably this problem belongs into the domain of molecular physics. For the moment we must content ourselves with the statement that ATP is needed as well for contraction as well as for relaxation. Both are colloidal reactions of the actin-myosin system, intensified by ATP.

Precipitation and dissociation are common phenomena of colloidal-chemistry and contraction and relaxation come in line with them. All the same the striking rate and extent and prompt reversibility of these reactions in actomyosin-ATP must impress even a colloidchemist, accustomed to the sight of these reactions and demand some further explanation.

MOMMAERTS has studied the quantitative relations of the dissociating effect of ATP. His problem was to find out what the smallest quantity of ATP is, necessary to cause complete dissociation of actomyosin in 0.6 *M* KCl. Actomyosin, under such conditions, is on the verge of dissociation. The result was that the actomyosin dissociated if there was 1 mol of ATP for every 100,000 g of actomyosin. MOMMAERTS worked with impure actomyosin, containing 15 % actin and some impurities so that his 100,000 g actomyosin contained about 70,000 g myosin. Dissociation was thus complete if there was one molecule of ATP for every myosin-unit of MW 70,000, which suggests that myosin is actually built of such particles.

The results of MOMMAERTS were corroborated by STRAUB (112). MOMMAERTS himself arrived at the same result by different methods (light-scattering, gelvolume). His results agree with WEBER and STÖVER who found that the myosin micel of MW 10^6 g is decomposed by strong urea into units of 10^5, while THE SVEDBERG showed that the probable MW for a protein-unit is not 100,000 but 70,000 g.

It is remarkable, that one such relatively small ATP-molecule should be able to change the properties of a particle of MW 70,000 g to such an extent. It has also been shown that $^1/_2$ K^+ adsorbed makes a very great difference to the properties of the particle of 17,600 g. One K^+ thus greatly changes the properties of 35,000 g myosin. All the same the K-binding curves are smooth and the properties of the actomyosin change gradually. This suggests that the single K-ions do not belong to such a limited area

which corresponds to MW 17,000, but belong to much bigger units and the single ions are the common property of these whole big units. It is probable that the K-ions take part with their electrons in the upmake of the common energy-levels of the particle, as described in a special article (150). If the particle has an MW of 10^6 g, as is probably true for myosin, and our curves are made to relate to such particles, then 5 K corresponds 300 K bound, 6 K to 360 K, and gradual transition becomes possible. The same may relate to ATP.

STRAUB (128) has shown that inorganic pyrophosphate has qualitatively the same dissociating effect as ATP though somewhat higher pyrophosphate-concentrations had to be employed to obtain the same effect. This shows that the pyrophosphate group is instrumental in the binding and the dissociat ng effect of ATP, while the rest of the molecule only modifies the action.

This effect of inorganic pyrophosphate is of interest, because myosin is unable to split this compound. This shows that the splitting of ATP is not involved in its dissociating effect. Since dissociation and contraction seem to be related phenomena, the activity of pyrophosphate suggests that the splitting of ATP is not involved in the mechanism of contraction either.

That pyrophosphate causes no contraction cannot be adduced as argument for the difference of the two reactions, precipitation and dissociation, for inorganic pyrophosphate dissociates actomyosin only at low temperature where contraction is very sluggish or there is no contraction at all.

Reversibility. The reactions described are reversible. So, for instance, if actomyosin-ATP is precipitated by KCl, the precipitate can be dissolved by increasing or decreasing the salt-concentration. The complex may be brought alternately to contraction and dissociation by alternately increasing and decreasing the salt-concentration. The reversibility of contraction also indicates that the splitting of ATP is not involved in this process.

As equilibrium reactions all these reactions will greatly depend on temperature. So, for instance, ATP, which readily precipitates actomyosin at room temperature, does not precipitate it at all at 0° and the actomyosin, precipitated at room temperature, may even dissolve, if cooled to 0°. Actomyosin is dissociated by inorganic pyrophosphate only at low temperature (0—6.5°); the reaction is reverted by bringing the system to 22°.

Mg has a double effect: in higher concentration it causes con-

traction, or superprecipitation of actomyosin-ATP, similarly to KCl. This is shown in Tab. VII. Below 0.001 *M* it has no such activity but is still capable of strongly enhancing the KCl-contraction. So, for instance, 0.02 *M* KCl gives only feeble contraction; if 0.0006 *M* $MgCl_2$ is present the contraction will be fast and strong.

Table VII.

M $MgCl_2$	0.01	0.005	0.0025	0.00125	0.0006	0
10"	x	x	xx	x	—	—
30"	xx	xx	xxx	xxx	—	—
60"	xxx	xxx	xxx	xxx	—	—
120"	xxx	xxx	xxx	xxx	—	—

0.125 % actomyosin, 0.05 % ATP. x = contraction, — = no change. Numbers mean the time elapsed between mixing and reading.

Another effect of Mg is that it greatly narrows the zone of KCl-contraction. This is illustrated by the Fig. 21 where X means contraction or precipitation. In this experiment 0.05 % ATP

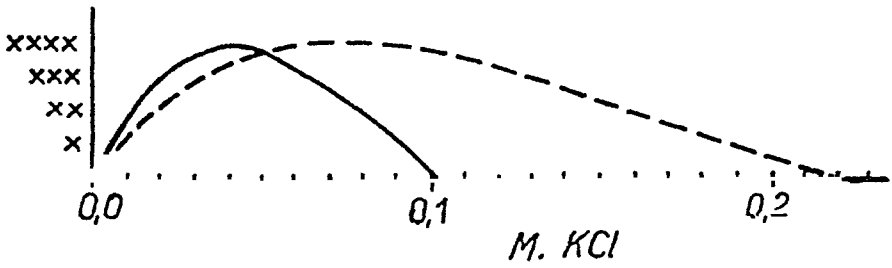


Fig. 21.

was present. In absence of $MgCl_2$ (broken line) the zone of precipitation extends from 0—0.2 *M*. In presence of 0.001 *M* $MgCl_2$ the zone is narrower, the curve steeper, which means that small changes in KCl concentrations have bigger effects and the protein answers the changes in concentration much sharper. With higher ATP concentration the precipitation zone would be still narrower. This relation of the ATP-concentration and the extent of the precipitation-zone is illustrated by the experiment reproduced in Tab. VIII. In this experiment the tubes contained 0.001 *M*

Table VIII.

M KCl	0.100	0.106	0.112	0.118	0.124	0.130	0.136	0.142	0.148
1 Min.	x	—	—	—	—	—	—	—	—
2 »	xxx	x	—	—	—	—	—	—	—
3 »	xxx	xxx	xx	x	—	—	—	—	—
5 »	xxx	xxx	xxx	xxx	x	—	—	—	—
15 »	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	x

$MgCl_2$, 0.125 % actomyosin and, at the begining, 0.05 % ATP. As the ATP is gradually split by the myosin the precipitation-zone extends more and more. In this experiment the concentration of KCl increases from tube to tube with no more than 0.006 *M*. It will be noted that the zone of inactivity is separated from the zone of strong contraction by no more than 0.012 *M* KCl. In presence of Mg the variation of the KCl concentrations by 0.012 *M* suffices thus to bring the relaxed actomyosin to contraction and *vice versa*. Decrease of the KCl-concentration by 0.012 *M* corresponds to a dilution of 10 %.

Evidently, these effects find their explanation in the Mg-myosinate-formation. Mg, even at 0.001 *M* concentration, saturates the myosin almost up to the isoelectric level. The K-adsorption of this Mg-myosinate is very steep.

It could be expected that Ca would have a similar effect, since Ca and Mg precipitate myosin in the same way. Contrary to this

Table IX.

M $CaCl_2$	0.01	0.005	0.0025	0.00125	0.0006	0.0003	0
60"	—	—	—	xx	xx	xxx	xxx
120"	—	—	—	xx	xx	xxx	xxx

0.08 *M* KCl, 13 % actomyosin, 0.05 % ATP. Signs as in Tab. VII.

expectation it was found that Ca, in itself, is unable to contract actomyosin-ATP. Ca is not only inactive, it inhibits the contracting effect of KCl, as shown by Tab. IX.

The inhibition is reversible. If the $CaCl_2$ is removed the acto-

myosin contracts again. But it is not even necessary to remove the $CaCl_2$. If $MgCl_2$ is added, the system contracts again. If in addition to 0.01 *M* $CaCl_2$, 0.0006 or 0.0012 *M* $MgCl_2$ is added to an actomyosin suspension, the colloid contracts as if no $CaCl_2$ would be present.

There is thus a definite antagonism between the action of Ca and K on one hand, and between Ca and Mg on the other. Mg, with its greater affinity to myosin competes more easily with Ca than does K. Whether the inhibitory effect of Ca is due to the inability of Ca-myosinate to contract or to an action of Ca on the actin, cannot be stated.

ADP, produced by splitting off one phosphate radicle from ATP by myosin, causes no contraction. It has no striking effect on the physical state of actomyosin whatever. As will be shown, it is not split by actomyosin either.

The ADP is converted into a reactive substance by the isomerase of BANGA (see there). Isomerase is active only in the presence of Mg. Our recrystallised myosin contains no Mg and no isomerase. Also the actin preparations of STRAUB are very poor in Mg and inactive even in the presence of isomerase. In the presence of Mg *and* isomerase ADP is split by actomyosin and will produce the same reaction (contraction, dissolution, etc.) as ATP. If both ATP and ADP act in the presence of Mg and isomerase the ADP will have the same effect as an ATP solution having half the concentration.

Isomerase, in the presence of Mg, will be of influence not only on the reaction given by ADP but also on that of ATP. Isomerase will enhance the effect of ATP and make contraction faster and stronger. This can be understood, because the isomerase, by converting ADP, not only doubles the number of active phosphate groups but also clears out an inactive reaction-product.

Watery muscle extract contains both Mg and isomerase. If it is dialysed for 24 hours it still contains isomerase and sufficient Mg to activate the isomerase. This Mg seems to be present in a non-diffusible form.

The watery extract is prepared by suspending the freshly minced muscle in equal parts of water, stirring it for ten minutes, pressing it out through a cloth and dialysing it against a great volume of water for 24 hours at 0°.

One part of this dialysed watery extract, if added to nine parts of actomyosin suspension, will make the ATP contraction faster

and stronger. In presence of this extract 0.02 *M* KCl will give maximal contraction (0.05 % ATP).

The actin-content greatly influences the reactivity of actomyosin. In Fig. 22 (GUBA 129) the viscosity of actomyosins of

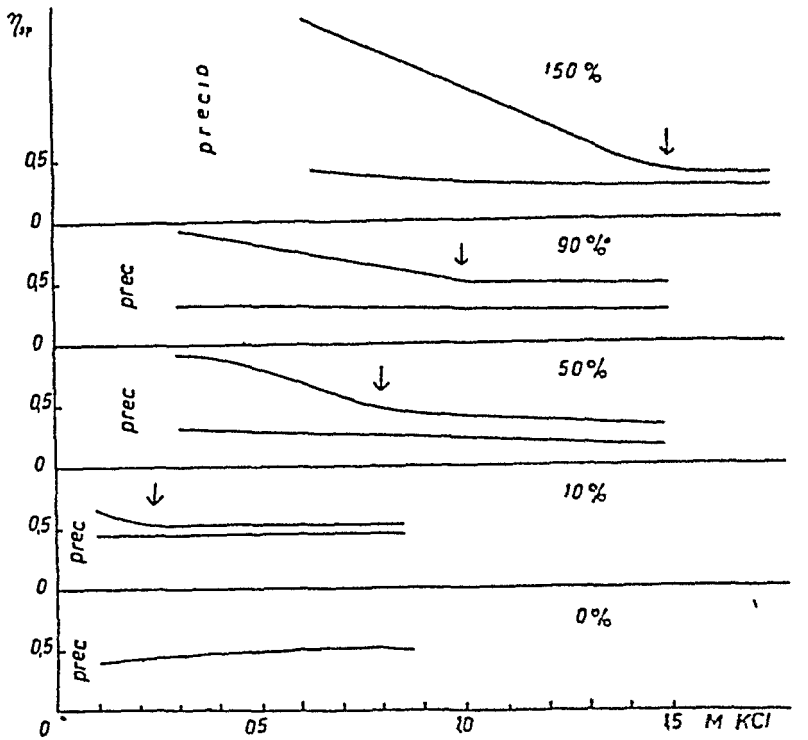


Fig. 22.

different activity is compared with and without ATP (0.015 %) at pH 7. The curves are analogous to those of Fig. 20. They show that the lower the activity (the lower the actin content) the less KCl is needed for complete dissociation (marked with an arrow). If the concentrations of KCl, necessary for complete dissociation, are plotted against activity, a straight line is obtained (Fig. 23).

These experiments show that actomyosins of different composition behave as individuals, which is even true for 10 % active actomyosin containing no more than 1.5 % actin.

The 10 and 90 % active actomyosins were prepared as such from muscle, the others from actin and myosin. All solutions contained 0.001 *M* $MgCl_2$.

The activity and the myosin: actin ratio. The formation of F-actomyosin from actin and myosin declares itself in the rise

of viscosity, the very viscous actomyosin being formed. The dissociation of this complex causes a corresponding drop of viscosity which has been termed the "activity". If increasing quantities of F-actin are gradually added to a certain quantity of myosin, in the beginning the activity rises roughly in proportion to the quantity of actin added. Later the curve flattens, reaches a maximum, and drops again on the further increase of actin concentration.

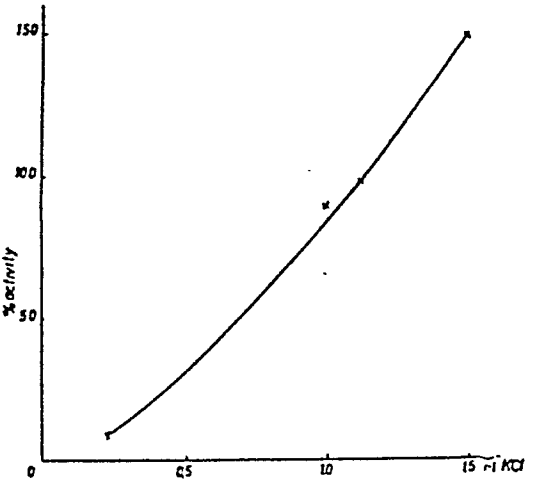


Fig. 23.

This is illustrated by the Figs. 24 and 25 (STRAUB 116). In the experiment, reproduced in Fig. 24, varying amounts of an actin solution were added to a myosin solution and the activity was measured. The curve shows the quantity of actin plotted against the activity. In the experiment of Fig. 25, the relative quantities of actin and myosin were varied within wide limits. In the curve the activity was

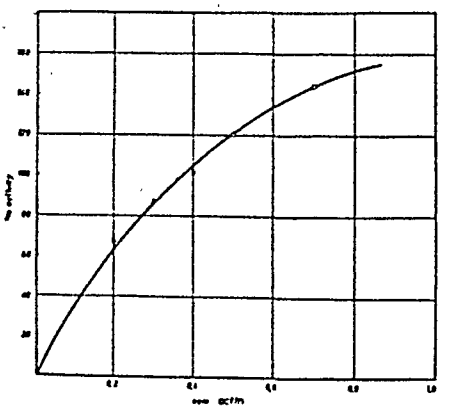


Fig. 24.

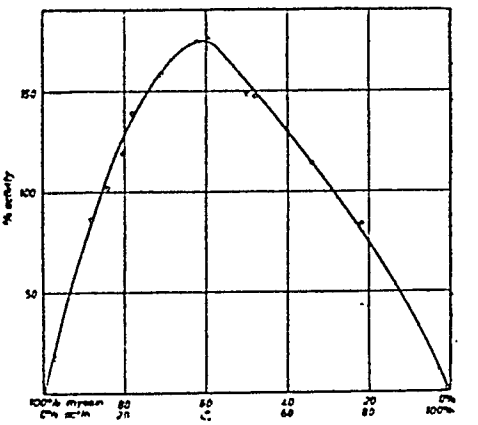


Fig. 25.

plotted against the actin-myosin ratio. It will be seen that the activity has a fairly sharp maximum at 170 %. At the beginning the curve is roughly linear and flattens as it approaches the maximum of activity. According to STRAUB, 2 mg of actin ac-

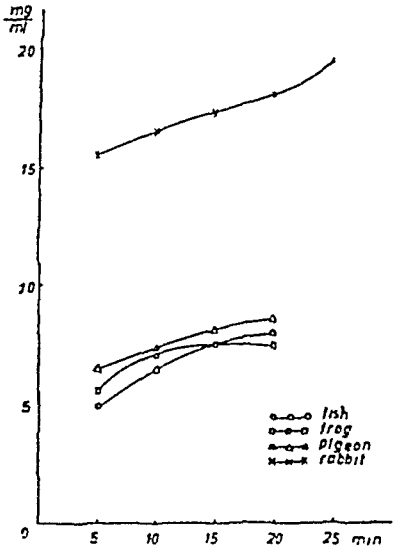


Fig. 26.

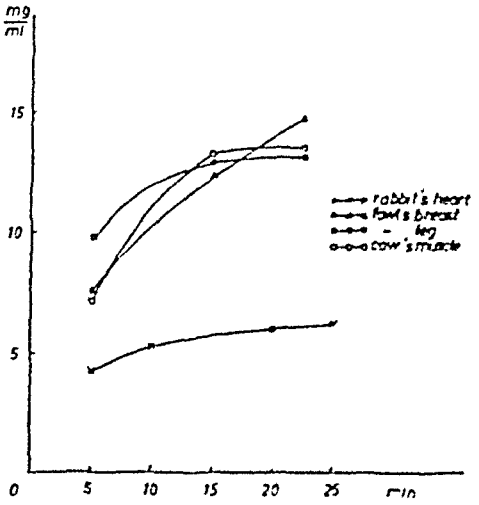


Fig. 27.

tivate 5 mg of myosin to the maximal 170 % activity. The 100 % active myosin B contains 1 part of actin to 5 parts of myosin.

According to BALENOVIÓ and STRAUB rabbit's muscle contains 25—30 mg actin per g. If we compare the more probable higher value, 30 mg, with the myosin content of muscle (80 mg per g) we find that the relation is very close to 2 : 5. Muscle contains thus maximally active actomyosin.

Comparative studies. If we mince the muscle of different animals and extract it with 0.6 *M* KCl (3 ml per g muscle) under constant stirring and take samples from time to time and estimate the quantity and the activity of the myosin, it will be found that the muscle of different animals and even the different muscles of the same animal, give off their myosin and actin with ease in different ways. Such experiments have been undertaken by F. GUBA whose results I will sum up by reproducing his curves (Fig. 26—29).

The two first curves give the quantity of the extracted myosin, the two last its activity. It will be seen that rabbit is an especially favourable object for the preparation of myosin, since it gives off its myosin rather easily and retains its actin rather strongly.

In fishes the actin is liberated readily and in 20 minutes a 120% active myosin is obtained.

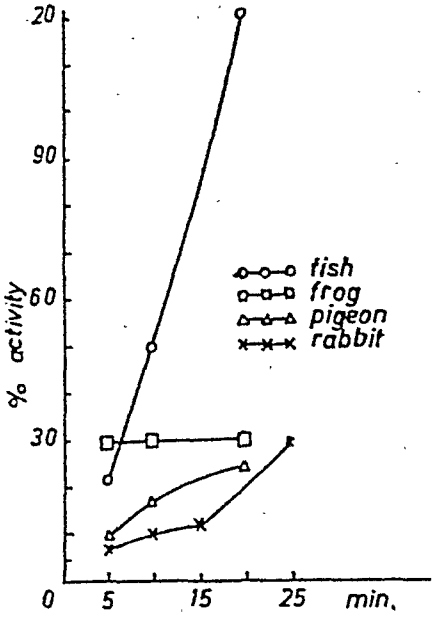


Fig. 28.

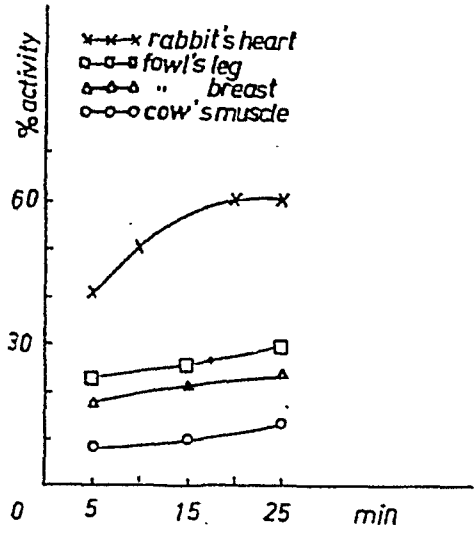


Fig. 29.

The F-G transformation of actin in contraction.

If to a strong KCl solution ATP, F-actin and myosin are added, a solution of dissociated actomyosin-ATP is obtained which contains the F-actin and the myosin-ATP side by side. If the substances are mixed in this order no chance is given for actomyosin-formation and contraction, and the resulting fluid has a strong DRF which corresponds to its actin-content. (The myosin does not contribute to the DRF, having none at higher KCl-concentration.)

If the same substances are mixed in a different order: if actin and myosin are put together, ATP and a little KCl are added, actomyosin will be formed which contracts. If the excess of KCl is added now, the actomyosin dissolves, and dissociates. The fluid will show no DRF whatever. That the actin was not destroyed, only depolymerised to G-actin, is shown by the fact that if time is given for the polymerisation, the F-actin is re-formed and the DRF appears.

This experiment shows that F-actin is broken up during contraction into G-actin. Contraction causes depolymerisation.

It has been shown that salts, without ATP, also cause a weak contraction and precipitation of actomyosin. Accordingly it is

found that if actomyosin is precipitated under the sole action of salts, it looses part of its DRF. (Actomyosin in precipitated condition, naturally, shows no DRF; it has to be dissolved to show DRF or the lack of it.)

G-actomyosin.

G-actomyosin has a low viscosity which equals that of free myosin. G-actomyosin is precipitated by salts much in the same way as F-actomyosin. On addition of ATP it does not contract but dissociates. G-actin forms thus no complex with myosin-ATP at any salt-concentration. Myosin greatly enhances the G—F transformation of G-actin. In presence of myosin G-actin will be polymerised in a few minutes time without addition of salt. If a small amount (0.05 *M*) KCl is added the transformation takes place within seconds. In presence of high salt-concentrations (0.6 *M* KCl) G-actomyosin is stable at 0° and myosin does not catalyse its G—F transformation: high salt-concentrations inhibit this catalysis.

That G-actomyosin is formed in presence of myosin, in spite of the unchanged viscosity, can be shown by adding F-actin to the mixture of G-actin and myosin. There will be no rise of viscosity the myosin being occupied by the G-actin present (STRAUB 116). In 0.6 *M* KCl at 0° G-actomyosin develops no DRF though free G-actin would rapidly be polymerised at this salt concentration; if ATP is added, the actomyosin dissociates, the actin polymerises and the DRF appears.

In presence of ATP myosin does not catalyse the G—F transformation, because myosin-ATP does not unite with G-actin. If a small amount of salt is present, G-actin, under these conditions, will polymerise slowly spontaneously and the resulting F-actin will unite with the myosin-ATP to contracted F-actomyosin-ATP. If no Mg is present the polymerisation will be very slow and precipitate will appear only after a longer period.

Myosin B.

If muscle is minced on the LATAPIE mincer, suspended in WEBER's fluid[1] (3 ml being taken per g of muscle), stirred for

[1] This solution contains 0.6 *M* KCl, 0.01 *M* Na_2CO_3 and 0.04 *M* $NaHCO_3$.

20 minutes and then centrifuged, the extract contains 15—20 mg of actomyosin of low (15—30 %) activity and viscosity. This is what has been called "A-myosin" by BANGA and the author. If this myosin is stored, its viscosity increases but slightly (owing to the decomposition of the ATP present).

If, however, the muscle suspension is not centrifuged but stored at 0°, within 24 hours it changes into a thick mass, which rather crawls than flows, from which the muscle particles cannot be separated any more by centrifugation. If the suspension is diluted with WEBER's fluid, centrifuged and the solution analysed, it will be found that the increased viscosity is not due to the greater concentration of myosin, but to the increase of its specific viscosity. If ATP is added now to this myosin the viscosity drops to relatively low values. This drop of viscosity has been termed "activity". The great number of our preparations always showed the same "activity". This myosin was called "myosin B" and its activity was termed "100 % activity".

Now we know that myosin B is an F-actomyosin containing 1 part of actin to 5 parts of myosin. Myosin B has now no special importance but at the beginning of our experiment it gave the first basis for quantitative measurements.

What happens in our muscle suspension during extraction is the following: the actomyosin of muscle is dissociated by the combined influence of the 0.6 *M* KCl and the ATP present, into actin and myosin. The myosin is extracted, taking a trace of actin with it. The bulk of actin is left behind in the muscle particles. If the suspension is stored at 0°, the ATP present is split in about three hours time. Now the dissolved myosin recombines with the actin and brings it into solution. Meanwhile also the binding of actin has been loosened up by the alkaline reaction. After the ATP has disappeared, the "activity" of the myosin begins to rise till it reaches 100 %, more and more actin being dissolved. If the suspension is stored longer, for several days, the activity begins to drop again, owing to the destruction of the actin which is rather sensitive to alkali.

Why the activity never exceeds 100 %, though the muscle contains 170 % active actomyosin, was studied by BALENOVIĆ and STRAUB. The main reason is that 5 parts of myosin seem to be able to bind only 1 part of actin sufficiently strongly to bring it into solution. But it was also found that the alkalinity of WEBER's fluid is sufficient to destroy actin. The structure-bound

actin is protected. Myosin also protects actin, 5 parts of myosin being capable of protecting 1 part of actin.

The myosin B can be precipitated by neutralisation and dilution. It can be redissolved in WEBER's fluid and reprecipitated. Often it will be a great advantage to add some ATP which causes superprecipitation, makes the precipitate roughfloccular and facilitates separation.

If we want to pull threads from our myosin B, its solution should not be too dilute because then, the threads will be too fragile. In this case we dilute the muscle suspension with 0.6 *M* KCl in the proportion of 100 : 80, and centrifuge. If a more concentrated myosin B solution is wanted, the undiluted myosin B can be liquified by the addition of a small amount of ATP, quickly centrifuged and poured off. After the ATP is split, the liquid sets to a gel which liquifies on shaking and can be pulled into threads. Till ATP is present no threads can be pulled.

In 0.6 *M* KCl myosin B gives a sticky, viscous, colloidal solution. On dialysis it sets into a gel which, in the absence of ATP, does not dissolve in 0.6 *M* even in 1.0 *M* KCl.

Actomyosin threads.

H. H. WEBER has shown (74) that a sufficiently strong "myosin" solution, if squirted in a thin jet into water, gelatinises in the form of a thread. Such a thread is an elongated piece of "myosin" gel.

Actomyosin threads are a very favourable material for the study of many features of contraction. They are a charming material too: their special charm lies partly in their superficial resemblance to the muscle fibre. They are less suited for the study of the solvatising action of salt or ATP because unspecific cohesive forces, developing in such a thread, inhibit dissolution. Diffusion also limits many reactions and complicates matters, the diffusion of added substances into the thread as well as the diffusion of the splitting-products of ATP out of the thread.

Research on threads was hitherto complicated by two circumstances. The one was that actin was not known and consequently undefined acto-myosins were used. The other was that the denaturing action of metals, present in common distilled water, was not recognised. The effect of metals on myosin is especially strong in the case of threads, great volumes of water being in

touch with a small quantity of the protein. Myosin binds heavy metals and is readily denatured by them. The properties of native and metal-denatured myosin threads are widely different. The latter can be stretched by 300 %, are elastic and their strength is considerable, comparable to that of muscle; they are enzymatically inactive. Native myosin cannot be stretched more than a few (10—15) percents and readily break showing hardly any resistance and split ATP. The research of WEBER has been done on metal-denatured threads, while M. and A. DUBUISSON and DUBUISSON and MONNIER worked with threads, denatured by drying.

Such denatured threads may be a most valuable material in certain lines of research but will give no information about native myosin.

The threads are pulled in dilute saline (0.05 *M* KCl), consist thus of salt-precipitated actomyosin. In salt-free water they loose their metal, swell and become unfit for work.

If an actomyosin thread is suspended in a fresh, watery extract of muscle[1] it contracts violently. Within half a minute it will be contracted to half, within 2—3 minutes to $^1/_3$ of its original length. At the same time the thread becomes proportionately thinner and becomes opaque. (Fig. 1.)

If the extract was not fresh and time was given to the ATP to decompose, the extract will be found to be inactive; its full activity will be restored by adding a suitable amount of ATP. ATP, however, if given alone in pure water, is inactive. A violent contraction can be obtained if, along with ATP, K (KCl) and Mg ($MgCl_2$) are added in a concentration, corresponding to the concentration of these ions in the extract.[2] This makes it evident that three substances of the extract were responsible for the contraction: ATP, K and Mg. A solution, containing 0.15 % ATP, 0.05 *M* KCl, and 0.001 *M* $MgCl_2$ will act in the same way as the extract.

The rate of the contraction of threads gives no idea about the rate of the elementary contraction process of micells, for the rate of contraction in threads is limited by the diffusion of the

[1] The watery extract is prepared by suspending the freshly minced muscle in an equivalent volume of water, stirring it for five minutes and pressing it out through a cloth. If the suspended muscle is only squeezed out the next day an inactive extract is obtained.

[2] Sometimes the contraction is so violent that the interior of the thread cannot keep pace with it and the outer layers break up like a crocodile-skin. Addition of a drop of watery muscle extract makes the contraction uniform.

reagents. In our experiments mostly threads of 0.2 mm diameter were used. Threads of half this diameter contract much faster. Threads of the dimension of the fibril must contract exceedingly fast. The rate of contraction can be sped up also by soaking the thread in the ATP-salt solution at 0°, at which temperature the thread does not contract. On bringing the thread to room-temperature a rapid contraction occurs.

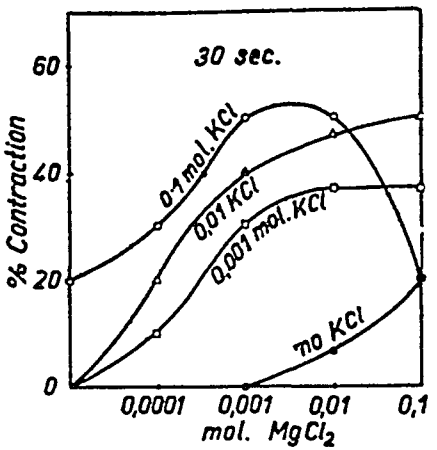


Fig. 30.

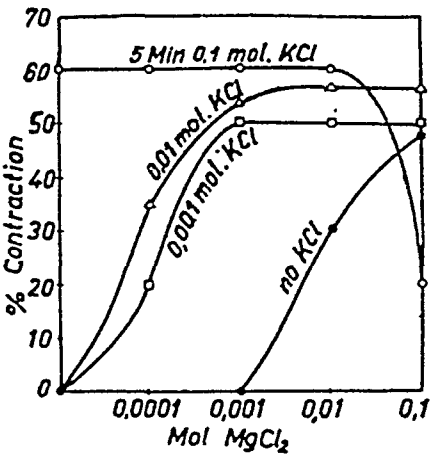


Fig. 31.

Contraction of myosin B threads in presence of 0.09 % ATP at varied KCl and $MgCl_2$ concentration. Readings made 30 seconds (Fig. 30) and 5 minutes (Fig. 31) after the addition of the ATP. The thread was bathed for 5 minutes in the salt solution previous to the addition of ATP.

The effect of salt-concentration on contraction can be studied with advantage on threads. The Fig. 30 and 31 (Erdös 114) show that the contraction of threads in $MgCl_2$ alone is always sluggish and can greatly be sped up by small concentrations (0.01 *M*) of KCl; also the contraction in (0.01 *M*) KCl is sluggish and can be sped up by small amounts (0.001 *M*) of Mg. In $CaCl_2$ there is no contraction at all and $CaCl_2$ (0.01 *M*) greatly slow down the KCl contraction. Small amounts of Mg (0.001 *M*), if added to the $CaCl_2$—KCl greatly speed up the contraction. These results agree with those obtained on suspensions.

In absence of Mg actomyosin threads contract with ATP up to 0.3 *M* KCl. At higher KCl concentrations the thread dissolves. 0.001 *M* Mg brings the limit of dissolution down to 0.19 *M* KCl. In presence of Mg there is a narrow zone of KCl concentrations (0.19—0.23) in which the thread neither contracts nor dissolves: is inactive. If the thread is soaked in such a KCl—Mg

mixture and ATP is added, there will be no change. If we now add a small quantity of water the thread contracts, the KCl concentration having been brought down into the activity range (GERENDÁS 113).

Contraction is reversible. If, for instance, a myosin B thread is brought to contraction in a solution containing 0.15 % ATP, 0.01 *M* KCl and 0.002 *M* $MgCl_2$, it will suffice to raise the KCl concentration to 0.025 *M* to cause the thread swell rapidly to its original dimension. If the KCl concentration is decreased again to its original value the thread contracts again. As contraction is promoted by Mg, so is relaxation. In absence of Mg the swelling is poor. If the KCl concentration is raised without Mg, the thread rather dissolves than swells.

Contracted threads can be made to relax also without ATP, but much higher salt concentrations and higher pH has to be used. It is immaterial in what way we hydrate the actomyosin, the result will be the same, relaxed actomyosin, which shows that contraction is but shrinking and relaxation but swelling or dissolution.

One would expect to be able to relax a contracted thread by suspending at, in absence of salts, in an ATP solution. This attempt was unsuccessful. The reason of the failure lies probably in diffusion, which makes that the K-phosphate, produced by the splitting of ATP, diffuses out slowly and acts as KCl. For the same reason strong ATP produces contraction without KCl.

The salt-effects described are not specific. KCl can be replaced by other alkali metals (109) while Mg can be replaced by Co and Mn (GERENDÁS 113).

There is one striking difference between the contraction of threads and muscle. While the thread contracts isodimensionally, that is to say, becomes shorter and thinner on contraction, muscle contracts anisodimensionally and becomes shorter and thicker. The difference is due to the orientation of micels which are arranged coaxially to the fibre in muscle. In the thread the micels are unoriented. There is only a very slight orientation, as indicated by the faint DR. (Accordingly also contraction, is not perfectly isodimensional either and the diminution of width lags somewhat behind the shortening).

There is one simple method of arranging the micels in a thread coaxially to the thread: stretching. Unfortunately, native myosin cannot be stretched, while denatured myosin does not contract.

M. GERENDÁS (113), who studied this problem, found that one can sail through by *partly* denaturing the thread. A partly denatured thread can be stretched to some extent and will contract to some extent. Lighter metals, like Zn, are the most suited. If the thread is bathed for 5 minutes in 0.001 *M* $ZnSO_4$, then washed out, it can be stretched, in some cases to 200 %, while the double refraction rises to $11.6.10^{-4}$ (Fig. 32). If the thread is kept for ten minutes in this stretched condition it sets and will not contract spontaneously any more. (At the beginning the stretching is elastic.) At 200 % stretching the orientation is far from maximal. The contraction too will be weak, no more than 40 % instead of 66 %. All the same, both orientation and contraction will be sufficiently strong to allow to study their effect.

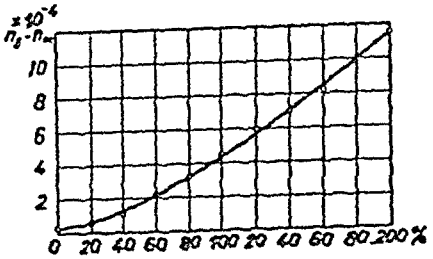


Fig. 32. The relation of stretching and DR. The thread was treated with $ZnSO_4$ (Quoted from GERENDÁS 113).

The experiments of GERENDÁS show that the contraction of such a thread is anisodimensional: the thread becomes shorter and thicker. To quote one example: while the thread became 30 % shorter it became 55 % thicker. At the same time its DR disappeared. Threads behave thus, in this respect, analogously to muscle, which, on contraction, becomes shorter and thicker and looses its DR.

Threads, suspended in pure glycerol, become extensible but their DR does not rise. Evidently the micels just shift along each other. Here again a compromise can be made. In 25 % glycerol the thread becomes stretchable, the DR rises and the contraction becomes anisodimensional. Here too DR is lost on contraction.

The anisodiametral contraction of oriented actomyosin threads proves that it is the actomyosin particles which change their shape in contraction, they become shorter and thicker. *The threads contract because the micels contract.* The loss of DR shows that they also bend or fold up.

Threads contract most vigorously at pH 6.5 but the pH dependence is not very sharp. (STRAUB, oral comm.) The contraction has a rather *flat* pH optimum.

Especial interest is attached to the limit of the contraction. This is 66 %. This value is very often reached but never exceeded.

(The few earlier readings of 70 % are undoubtedly due to methodical error.) If the actomyosin content of such a thread is calculated, it will be found to consist of about a 50 % of actomyosin and contains thus but 50 % of water. 96 out of the 98 % of the water, which made up the actomyosin-containing thread have been pressed out. Since there must be some intermicellar spaces left, which must be filled with water, we may conclude that *actomyosin, precipitated by salt and ATP, is completely dehydrated.*

T. ERDÖS (133) has studied the relation between contractibility and actin content. He found that threads of 40—170 % activity contract equally fast and strongly. Between 0—40 % activity, the contractibility is proportional to the myosin content. It is rather striking that a thread of 40 % activity, containing 7.5 % actin, should still contract maximally.

Threads, prepared from synthetic actomyosin or from myosin B, contract in the same way, except that the actomyosin threads do not contract with ADP, while myosin B threads do. The difference is due to the absence of isomerase in actomyosin and its presence in myosin B. By adding a small quantity of watery muscle extract, the actomyosin threads can be made to react on ADP too. If the myosin B thread is washed with Mg-free KCl solution, it soon looses its reactivity towards ADP. This is not due to the removal of isomerase, which is strongly adsorbed, but to the removal of Mg.

AMP has no marked influence on threads at all.

Technical remarks.

Threads. It is most convenient to work with a 1—2 % actomyosin solution, 0.5—0.6 *M* KCl being used as solvent. Threads, prepared from more dilute solutions, are too fragile, while those prepared from stronger solutions are less contractile. Solutions, stronger than 5 %, are too viscous. The solution should contain no ATP, in the presence of which the actomyosin behaves as myosin.

It was found to be the most convenient to work with threads of 2—3 mm length and 0.2 mm diameter. Thicker threads are too sluggish, thinner ones contract too fast and have too strong a tendency to curl up before they contract. The threads were observed under the microscope, suspended in fluid on big hollow-

ground slides. Low power was used so that the whole length of the thread was visible and was measured by an ocular-micrometer. Care was taken to make readings only when the thread was lying horizontally, otherwise the parallaxe caused considerable error. (The thread lies horizontally when its whole length is clearly visible without moving the micrometer screw.) The thread should swim or lie freely and should not stick to the glass.

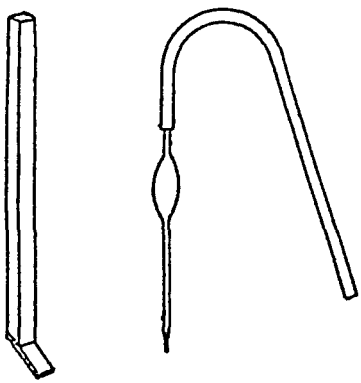


Fig. 33.

The threads were transferred from one fluid into the other by means of a small home-made celluloid spatula (Fig. 33, left). Actomyosin does not stick to celluloid. The width of the spatula corresponded to the desired length of the thread. The spatula was pushed under the thread and lifted. By cutting along the sides, a piece of the desired length was obtained.

For many purposes hand-made threads will do: the actomyosin solution is sucked up in a glass tube one end of which is pulled out to a capillary which constantly narrows towards the end. By breaking off more and more of the end an opening of the desired size can be produced. The wide end is armed with a rubber tube. By blowing into the tube we provide a constant outflow, while the capillary end is dipped into a 0.05 *M* KCl solution and the tube is moved by hand to the right and left. A wide and not too shallow basin should be used.

The diameter of the thread depends on the diameter of the opening of the capillary, the rate of outflow, the viscosity of the solution and the speed of motion. With some practice fairly uniform threads will be prepared.

For comparative and quantitative studies such threads will not be sufficiently uniform. In this case the threads have to be pulled mechanically. An apparatus for this purpose was described by M. Gerendás (113) and M. and A. Dubuisson.

In the apparatus of Gerendás (Fig. 34) the capillary (K) is fixed, the basin (Gl) with the fluid (Fl) is moved, being rotated by a motor (Em). The pressure is established by the rubber pump and stabilised by the rubber ball (B). The actomyosin is placed into the container (Ap). In Dubuisson's apparatus the fluid is fixed and the capillary moves.

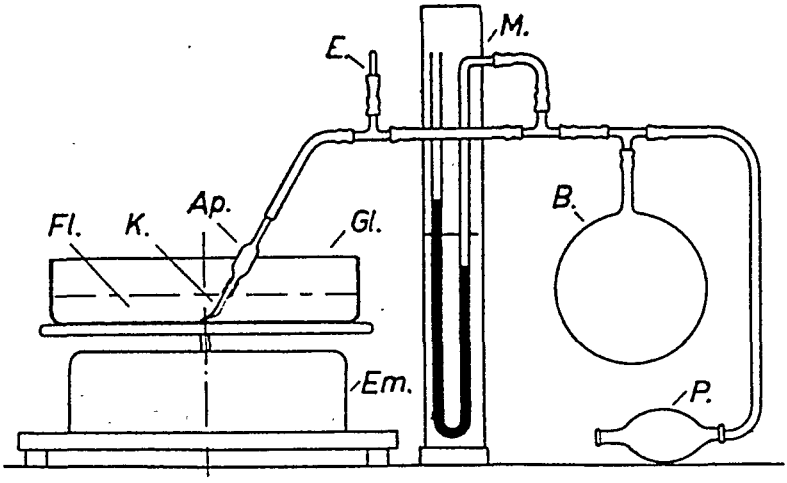


Fig. 34.

No threads can be obtained by this method from myosin. A salt solution of myosin, if squirted into water, dissolves or flocculates. Threads can be prepared from myosin by dialysing the crystalline myosin. After the myosin has lost its metal it swells up to a gel. This gel, if squirted into 0.02 *M* KCl, will form threads. Air-pressure is insufficient to press this gel through a capillary. It can be pressed out with a piston fitting into the wide part of the tube with capillary end.

Difficulty will be experienced with pulling threads from actomyosin with very low actin content. Neutralisation of the solution and low salt content may help. We may also use 25 % glycerol to pull the thread in.

If we want to observe contraction, produced by the addition of ATP, care should be taken for its homogeneous distribution. For this reason the ATP, in our experiments, was squirted in from a micropipette, by blowing into its rubber tube (Fig. 33). Mixing was completed by blowing on the surface of the fluid from the empty pipette. If the ATP reaches the thread from one side, this will curl up.

Suspensions. A dilute actomyosin solution (0.01 %) is precipitated by salts and ATP. A more concentrated solution (0.1 %) may react, according to conditions, with "superprecipitation" or contraction. If the actomyosin forms a homogeneous system then it will react with contraction. If it is broken up to discontinuous flocculi, then the single flocculus will contract and we obtain superprecipitation. So if we want to observe superprecipitation

we should produce a discontinuous suspension, if we want to see contraction we must be careful not to break up the colloid.

To give an example: let us start with 2 % actomyosin dissolved in 0.5 *M* KCl. If we precipitate the actomyosin by diluting the solution and resuspend the precipitate, we will have a discontinuous suspension, consisting of flocculi. Addition of salt and ATP will make the precipitation more intense and the flocculi granular, thus cause superprecipitation. But if we add the ATP to the undiluted salt solution of actomyosin, this causes the dissociation of the protein and herewith a complete homogenisation. If water is now suddenly added in sufficient amount to bring the KCl concentration down to 0.1 *M*, the colloid forms a homogeneous solution and contracts. Another example: if we want to see contraction in a system, produced by mixing myosin, actin, salt and ATP, much will depend on the order, in which the single constituents are mixed. If myosin is mixed first with salt, then actin and ATP are added, we will not see good contraction because the salt precipitates the myosin and the actomyosin formed will not be homogeneous. If, however, after adding salt to myosin, we add the ATP, this dissolves the myosin-precipitate. If we now add the actin at the end and mix the fluid with one energetic shake, the system will contract.

Observing contraction it is advisable not to take wide tubes and high columns of fluid because the cohesion of the colloid is small and might be insufficient to pull the big mass together against the resistance of the water, and the colloid will break up into flocculi. Tubes of 10 mm diameter are convenient.

If we work with actomyosin prepared from actin and myosin and our problem is whether a certain salt- or ATP-concentration precipitates or dissolves the complex protein, we may proceed in two different ways. We may prepare the actomyosin suspension and see, whether it dissolves, or we may add the salt and ATP to the actin (or myosin) and add the myosin (or actin) subsequently and see whether a precipitate will be formed or not. Theoretically both methods give identical results since all these reactions are reversible. The latter method, however, will often give more accurate results, because actomyosin, if kept in the gel form or in a precipitated condition, very quickly develops unspecific cohesive links which inhibit or prevent dissolution.

A few words may also be said about the method of observing precipitation. It would seem most logical to use side illumination with a black background. This method, however, often gives paradoxical results and precipitate-formations goes hand in hand with decrease of luminosity.

Reliable results were obtained if the fluid was observed before a black background and illuminated from behind at a very small angle. In order to convert this principle into a simple method, the test tubes were placed into a waterbath with glass walls. On the backwall a screen was fixed with black strips. The screen was illuminated from behind (Fig. 35). The strips represent the

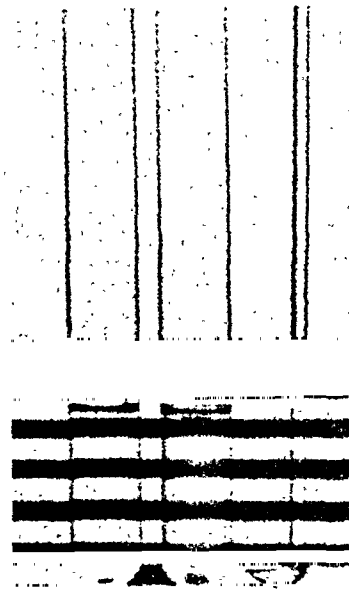


Fig. 35.

black background, while the light falls in at a small angle from between the strips. Precipitate-formation makes the black strips gray or hazy. The ATP was introduced with a small glass spoon (Fig. 35). This spoon, if dipped quickly once or twice into the solution, provided the necessary mixing.

Suspensions are less suited for these studies of the kinetics of contraction than are threads. Contraction, in suspensions, has a latent period, which may last from 10—60 seconds. In thread, with their closed packing, there is no such latency. Here contraction is limited by diffusion.

PART IV

Experiments on Muscle.

Slices.

We may ask how far the reported observations on myosin and actin have any bearing on the function of muscle.

The experimental material presented suggests that muscular contraction is simply a reaction of the actomyosin micel to the influence of salts and ATP. If this is true, then it does not matter how far we destroy the finer structure of the muscle, as long as we leave the actomyosin intact and add salts and ATP, the muscle has to contract.

In order to test this point the broad and flat neck-muscle of the rabbit was cut nto strips. This muscle consists of sheets of parellel-running fibres; the strips were placed into distilled water and kept in the ice-box for 2—3 days during which time the water was exchanged repeatedly. The muscle slowly contracted and was stretched out again to its original length till no more contraction occurred. Then the muscle was cut on the freezing microtome into slices about 0.1 mm thick, care being taken that the cut was parallel to its surface. The single slices consisted of one sheet of fibres running along their whole length. The slices were placed in distilled water.[1]

If such a slice is placed on a slide covered with 0.05 *M* KCl, containing 0.001 *M* $MgCl_2$ and then a drop of 1 % ATP is added the slice contracts violently to about $^1/_3$—$^1/_5$ of its original length. The single fibres become shorter and thicker. This shows that destruction of the finer structure by freezing, thawing and thorough extraction, does not abolish the contractility.

[1] If the fresh, unextracted muscle is frozen, on thawing it contracts.

Fibres.

T. ERDÖS (unpublished) has made minced muscle the object of his studies. The deep back-muscle of the rabbit was cut out as free of fasciae as possible and minced in the LATAPIE mincer. (This is a meat mincer with relatively small — 1 mm — holes.) If a small quantity of the mince is suspended in distilled water and is observed under the microscope it will be found to consist chiefly of isolated, uncontracted somewhat swollen muscle fibres. Only here and there does a fibre contract to a small extent.

If, instead of distilled water, the muscle is suspended in 0.02 *M* KCl, the fibres contract energetically to $^1/_3$—$^1/_5$ of their original length. If the KCl concentration is varied, contraction is obtained up to 0.2 *M*. At higher concentration (0.25 *M*) there is no more contraction.[1] The muscle fibres behave thus similarly to an actomyosin thread. As will be remembered, actomyosin does not contract on addition of ATP in the absence of salts, it contracts in 0.02—0.2 *M* KCl but does not do so in 0.25 *M* KCl.

If the fibres are suspended in a great volume of distilled water, the salts and the ATP will be soon washed out and the fibres will contract in salt solution only on addition of ATP. This they will do even after days of storage in water.

Rigor and contracture.

It has been shown that at a given salt-concentration the physical condition of actomyosin depends on the concentration of the ATP present. In the absence of ATP actomyosin is insoluble in KCl up to 0.4 *M*. If the actomyosin has gelatinised it is insoluble even in 1 *M* KCl. It can be rendered soluble by the addition of ATP.

Rigor mortis. ERDÖS (132) studied the question whether the relaxed state of muscle is actually dependent on the ATP concentration and whether there is any relation between the degree of relaxation (softness) of muscle and the ATP concentration. He compared hardness and ATP concentration of muscle after death.

The results are summed up in his two figures, Fig. 36 and 37. In the first the degree of rigor and the ATP concentration of the muscle are compared. It will be seen that they are almost mirror

[1] If ATP is added to the saline, the fibres contract up to 0.4 *M* KCl. The same is true for slices. Added ATP acts, for same reason, differently to the fibres' own ATP.

images and run exactly antiparallel. The second curve shows that parallel with the development of rigor the actomyosin becomes insoluble but can be rendered soluble again by the addition of

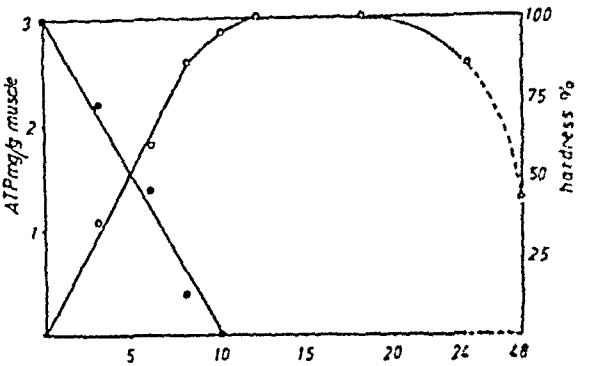


Fig. 36. ATP content during development of rigor mortis, in rabbit muscle.
● Hardness. 0 % = fresh muscle, 100 % = maximal rigor.
○ mg ATP per g of muscle.

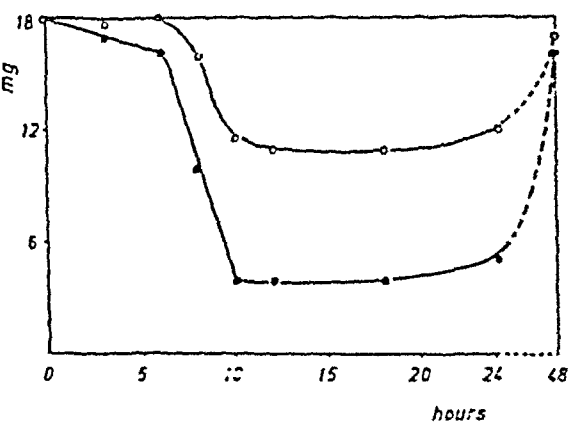


Fig. 37. Solubility of myosin during the development of rigor mortis.
● Myosin mg/ml extracted with 3 vols. of 0.6 *M* KCl.
○ Myosin mg/ml extracted with 3 vols. of 0.6 *M* KCl in presence of 0,3 % ATP.

ATP. Rigor and insolubility are thus the different expressions or consequences of one and the same condition, the lack of ATP.

As is generally known, the rigor relaxes after some time. This is shown by the declining end of the upper curve of Fig. 36. The muscle relaxes and at the same time the myosin becomes soluble though there is no ATP.

The explanation of this phenomenon is given by the analysis of the extracted actomyosin. At the beginning of the experiment, before rigor sets in, actomyosin dissociates under influence of the

ATP; the myosin is extracted, while the actin is retained by the structure. At the end of the experiment, when the rigor ceases, actomyosin is extracted. This shows that the relaxation is due to the final disorganisation, the actin being released by the structure.

Contractures. A less close parallel can be expected between hardness of muscle and ATP concentration in other forms of con-

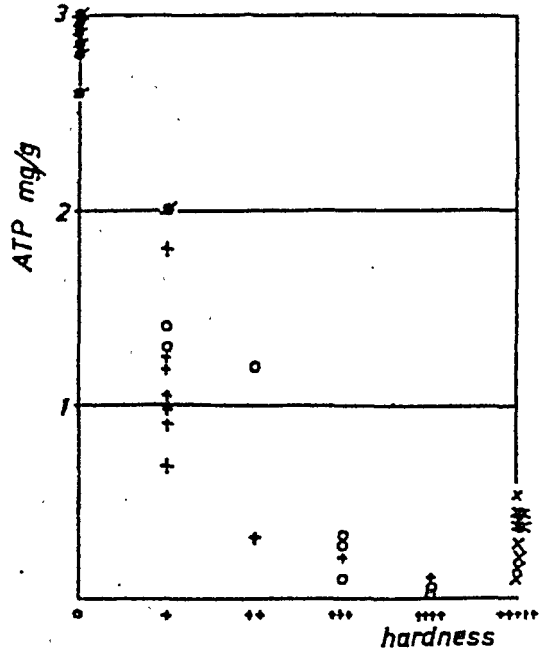


Fig. 38. ATP content and hardness of frog muscle, in different forms of contractures.
○ is the hardness of fresh muscle. +++++++ is the hardness of muscle in maximal rigor. ● Fresh muscle. O Muscle 24—48 hours after decapitation. (At 18°—20°.) + Electrically stimulated muscle. × Isolated gastrocnemius exposed to chloroform vapours for 12 mins. left leg resting, ● stimulated leg of frog poisoned with monojodoacetate.
The hardness was measured *in situ*. The same muscles were used for ATP determination.

tracture. Contracture can be produced by very different means, so for instance, by monojodoacetate, prolonged stimulation, coffeine, chloroform-vapour. If the hardness of the resting muscle is measured, it will be found that contracture does not develop suddenly but that the hardness of the muscle increases gradually. ERDÖS has measured the ATP content of muscle and the solubility of myosin also in these conditions and found the same relation as in *rigor mortis* with the difference, that the results were more scattered (Fig. 38).

These experiments, put together, suggest that the ATP of muscle has a decisive influence on the physical state of the resting muscle and its actomyosin. The ATP is necessary for the relaxed state. The normal ATP level seems to be necessary to keep the muscle in a fully relaxed state; as soon as the ATP content begins to drop, the hardness of the muscle increases.

The experiments of ERDÖS suggest this further generalisation: if the ATP is decomposed in a muscle, which is not excitable any more, the insoluble, F-actomyosin gel is formed which makes the muscle stiff and hard (*rigor mortis*). In this state there is only a slight salt-contraction. If, however, the ATP is decomposed in a muscle which is still excitable, the muscle will be unable to relax and we shall obtain contracted actomyosin and herewith not rigor but contracture. *In vitro*, under certain conditions (Tab. VIII), decomposition of ATP entails contraction.

As mentioned in the introduction to myosin, the *post mortem* decrease of the solubility of myosin as well as the increasing insolubility after contraction under the influence of monojodoacetate, have been noted and studied by other investigators. The existence of actin and the action of ATP on actomyosin, not being known, the result remained necessarily unexplained.

The experiments reported afford an explanation of the therapeutic activity of ATP. The pathological conditions, in which favourable therapeutic effects were obtained with ATP, are due to the spastic contraction of smooth muscle cells (angina pectoris, certain vasospastic gangrenes and dysmenorrhea). The fact, that ATP causes relaxation indicates that the disturbance was due to the lack of this substance.

PART V.

Enzymic Functions.

The ATP-ase action of myosin.

JACOBSEN (1), BARRENSCHEN and LÁNG (2) demonstrated the existence of a phosphate-splitting enzyme in animal tissues which exhibited great substrate specificity towards ATP. This enzyme, called ATP-ase, liberates two inorganic phosphates from ATP thus forming AMP. Later T. SATOH (3) held the view that the dephosphorylation of ATP is accomplished by two enzymes, one is a pyrophosphatase, producing ADP, the other is a phosphomonoesterase. One of them needs an activator while the other, working at pH 9.0, needs Mg ions. LOHMANN (4) found that in washed crab's muscle only one phosphate is split off from ATP. He found also that even in muscle tissues, in which the ATP is broken down to AMP, the washing of the muscle renders it incapable of splitting more than one phosphate group, and that the addition of Mg restores the full activity.

According to ENGELHARDT and LJUBIMOWA the phosphatase-action of muscle is due to myosin, which splits off one phosphate from ATP. The second phosphate is split off by a water-soluble enzyme.[1]

K. LAKI (126) prepared fine suspensions of muscle, and found that the insoluble muscle particle in itself is only capable of splitting off one phosphate group from the ATP molecule. The splitting of a second phosphate group is due to the joint action of the insoluble muscle particle, a soluble protein and Mg ions.

The enzymic activity of crystallised myosin was studied by I. BANGA (135). She found that the first phosphate group of ATP was split off by twice recrystallised myosin with equal intensity as by earlier impure preparations.

[1] Quoted from LAKI.

At the earlier stages of her research, when still working with impure and undefined actomyosins, I. BANGA (110) found that the splitting of ATP by "myosin" depends on the presence of ions. All the salts tested: LiCl, NaCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $CoCl_2$, $MnCl_2$, $ZnSO_4$, $NiCl_2$ activated myosin, provided that they did not denature it as did $AlCl_3$ or $CuCl_2$. These experiments of BANGA were corroborated by D. M. NEEDHAM.

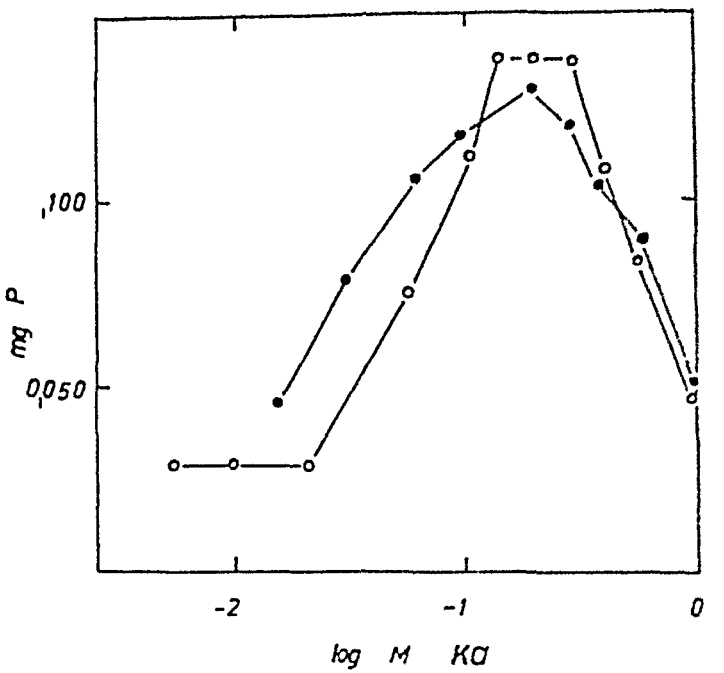


Fig. 39. Effect of KCl on the phosphatase activity of myosin and actomyosin. Formation of inorganic P determined by the method of FISKE and SUBBAROW. 1 mg myosin or 1 mg myosin plus 0.3 mg actin in 3 ml of water, 3.6 mg ATP as neutral K salt. Incubation for 5 min. at 38°.
Points = myosin, circles = actomyosin.

BANGA repeated these experiments later with pure recrystallised myosin (135, 136). She found that crystalline myosin is capable of splitting off only one phosphate from ATP. This function greatly depends on the presence of ions and is greatly activated by KCl. Very probably myosin is quite inactive in the entire absence of salts. Unfortunately this cannot be tested because ATP itself is a salt, if added at neutral reaction. At any rate the function is greatly accelerated by KCl with an optimum at 0.05 *M* (Fig. 39). Myosin and actomyosin, as the Fig. shows, are activated in the same way.

In terms of metal-myosinate this means that free, negatively charged myosin is inactive. Only metal-myosinate is active. The maximum of activity is about 0.2 *M* KCl, where myosin binds 6 K^+ per unit-weight.

$CaCl_2$ had an effect, similar to KCl. It greatly enhanced the activity of myosin and actomyosin (Fig. 40—41).

We could expect Mg to do the same as Ca. BANGA found that,

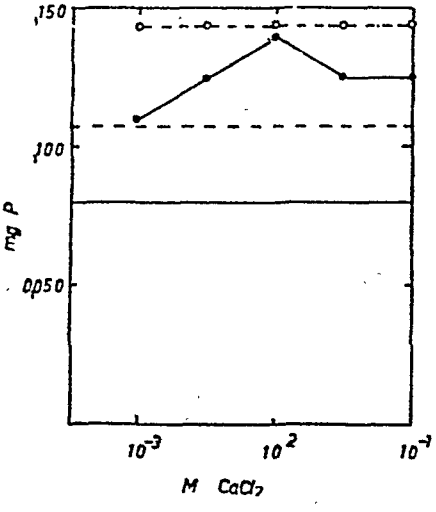


Fig. 40. Effect of $CaCl_2$ on the phosphatase activity of myosin in the presence of 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $CaCl_2$.

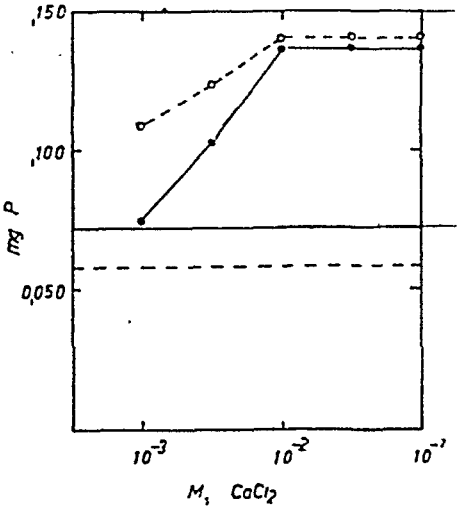


Fig. 41. Effect of $CaCl_2$ on the phosphatase activity of myosin in the presence of 0.1 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $CaCl_2$.

contrary to this expectation, Mg, even in very small concentrations, almost completely inhibited the enzymic activity of myosin and accelerated that of actomyosin (Fig. 42). Alkalimyosinates are thus active and so is Ca-myosinate; Mg-myosinate is completely inactive. Actomyosin-Mg, on the other hand, is fully active.

The action of Mg on actomyosin depended on the concentration of the KCl present. (Fig. 43—44.) At a low KCl concentration (0.01 *M*) or in the absence of KCl, $MgCl_2$ enhanced the activity, at a higher (0.1 *M*) KCl concentration Mg inhibited the activity. The explanation of this paradoxical behaviour is simple. In presence of higher KCl concentrations actomyosin dissociates into actin and myosin and the activity of myosin is inhibited by the Mg. As will be noted on the curve, only high concentrations of Mg inhibit the action of actomyosin, which cause a dissociation

in presence of ATP. $CaCl_2$ has the same action on myosin and actomyosin, thus its activity is independent of the KCl concentration (Fig. 40, 41).

The combined effect of Ca and Mg was found to be inhibitory in both cases, in the case of myosin as well as in that of actomyosin. (Fig. 45.)

The ADP isomerase of Banga.

If actomyosin acts on ATP, one phosphate group is split off and the reaction stops. If a small amount of watery extract of muscle is added, the reaction goes on and more phosphate is liberated. The extract alone is inactive.

If ADP, formed from ATP by the detachment of one phosphate, is isolated and added to actomyosin, there will be no change. If together with actomyosin a small amount of watery muscle-extract is added, a second phosphate is split off. The question arises: what is the mechanism of the action of the watery muscle-extract?

If the ADP was incubated for a short time with watery muscle-extract, the protein destroyed and the mixture added to actomyosin, again there was a further liberation of phosphate. Evidently, the watery extract did something to the ADP which made it reactive again. The most simple explanation would have been to ascribe this effect to KALCKAR's dismutase. This author described an enzyme which is capable of dismutating ADP into ATP and AMP. The experiments, however, showed that dismutation could not be responsible for the effect for there was no dismutation at all: there was no ATP formed since the reaction product was not attacked by pure myosin, only by actomyosin; there was no adenylic acid formed either since desamidase caused no desamidation. Furthermore the splitting of the reaction-product showed quite different qualities as the splitting of ATP (see below). Dismutation could thus be excluded. The most probable explanation seemed to be that the catalyst, present in the extract, induced some intramolecular rearrangement within the ADP molecule. This conclusion was supported by the colloidal reactions which showed that the ADP causes contraction only after having been acted upon by the watery extract. This suggested that under the influence of this extract the active pyrophosphate configuration was restored. For these reasons BANGA

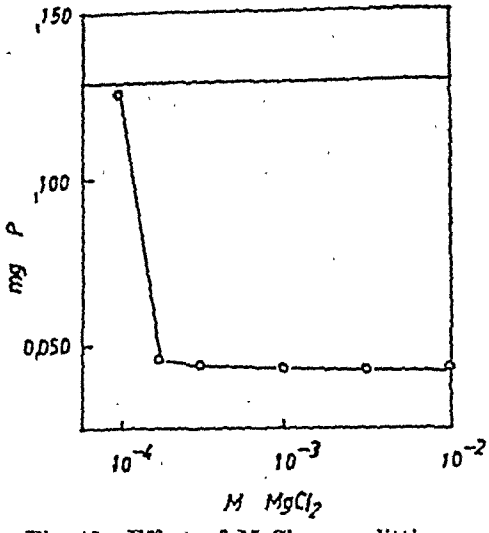


Fig. 42. Effect of $MgCl_2$ on splitting of ATP by crystallised myosin. mg P split off at 38° in 5 min. from a mixture of 1 mg myosin, 0.1 *M* KCl, 3.6 mg ATP, and varying concentrations of $MgCl_2$. Total volume 3 ml.

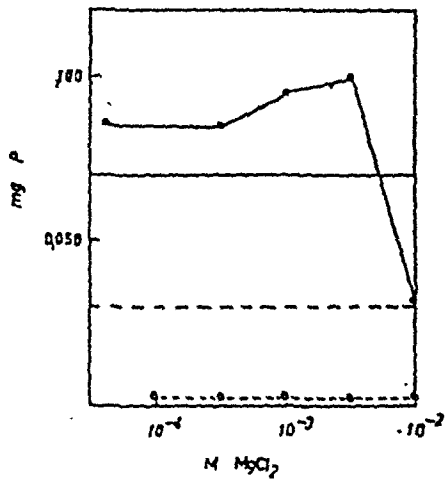


Fig. 43. Effect of $MgCl_2$ on the phosphatase activity of myosin in the presence of 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $MgCl_2$.

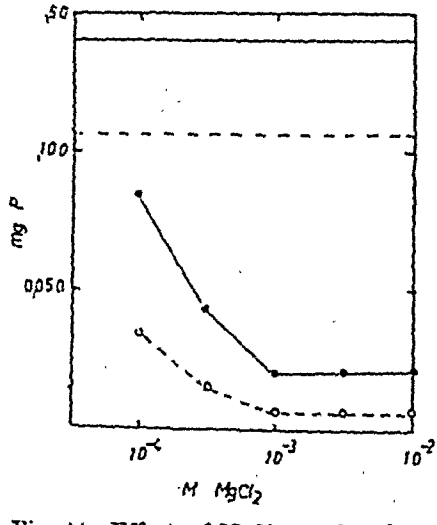


Fig. 44. Effect of $MgCl_2$ on the phosphatase activity of myosin in the presence of 0.1 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $MgCl_2$.

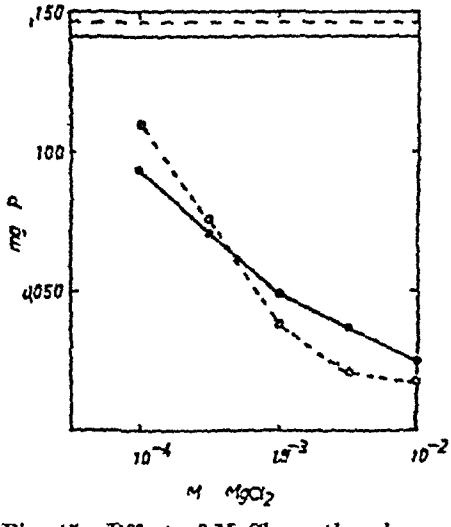


Fig. 45. Effect of $MgCl_2$ on the phosphatase activity of myosin and actomyosin in presence of 0.01 *M* $CaCl_2$ and 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The lines on the top give the phosphatase activity in absence of $MgCl_2$.

called the catalyst ADP-isomerase, and supposed that its action was to induce some intramolecular rearrangement and isomerise the ADP.

The isomerase was purified in the following way: "Freshly minced muscle of rabbit was suspended in 0.1 *M* KCl, 1.5 ml being taken per g of the muscle. Two hours later the insoluble parts were removed by centrifugation. Ammonium sulfate was added to 0.5 saturation and the precipitate discarded. The liquid was neutralised and ammonium sulfate added to 0.7 saturation. The resulting precipitate contained the ADP-isomerase. It was dissolved in 0.1 *M* borate buffer of pH 8.5, dialysed for 24 hours and the insoluble parts removed by centrifugation. The isomerase was absorbed to γ $Al(OH)_3$. From this solution the isomerase precipitated between 0.5—0.6 saturation of ammonium-sulfate. 50 γ of this protein in 3 ml activated the splitting of ADP by actomyosin."

The product was 50 times more active than the extract, if calculated pro g protein.

The activity of the isomerase depends on the presence of Mg. Traces of Mg are sufficient to activate the enzyme. Even as small traces of Mg, as are retained after dialysis, are sufficient to cause activation. This explains the finding of LAKI, according to which the splitting of the second phosphate by his muscle preparations was inhibited by pyrophosphate. In all probability this effect of pyrophosphate was due to the binding of the traces of Mg, retained by the proteins, which can be removed by special methods only.

The isomerase can be precipitated by trichloro-acetic acid without loss and can be heated to 100° in presence of 0.15 *M* HCl for ten minutes without loss of activity; it resembles KALCKAR's dismutase in this respect.

ADP I, ADP II and ADP III.

In her last, unpublished paper, BANGA showed that, according to conditions, three different ADP:s can be obtained from ATP under action of myosin. She called them ADP I, ADP II and ADP III.

If myosin acts on ATP phosphate will be liberated. BANGA followed the rate of the phosphate liberation (Fig. 46 lower curve) and found that about half of one of the readily hydrolysable phosphate groups is split off very fast and then the reaction slows down to be completed only after a long period, 12 hours or so. By "completion" is meant the splitting off of the one of the two readily hydrolysable phosphates.[1]

[1] ATP has two phosphate groups which can readily be hydrolysed by *N* HCl at 100°. The third phosphate group is stable and can be detached only by protracted hydrolysis.

If myosin acts in the presence of "isomerase", the reaction goes on till about half of the second labile phosphate is split off, and slows down afterwards. "Isomerase" is the enzyme preparation of BANGA described in the previous chapter. Whether the catalyst, present in this preparation, which makes the second phos-

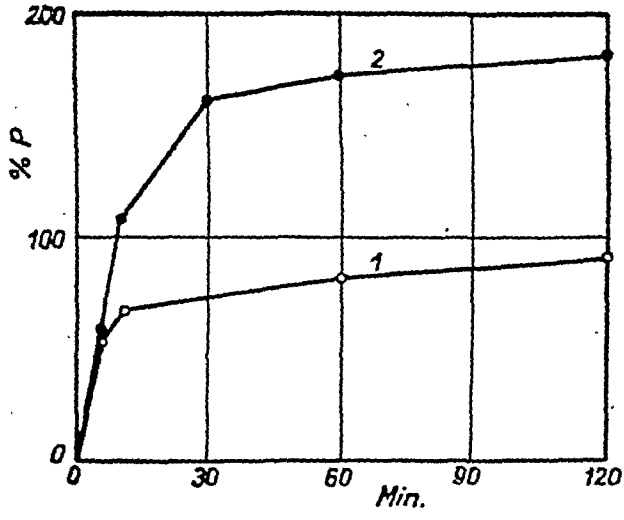


Fig. 46.

phate accessible, is the same as the one which acts on ADP, is left open. Both resist boiling in acid and can be precipitated inactive form by trichloroacetic acid.

ADP I is obtained if myosin acts on ATP in the presence of "isomerase" and the mixture is incubated for a short period, 10 minutes or so. ADP II is obtained if the same reaction is done in absence of isomerase. ADP III is obtained if myosin acts on ATP in the absence of isomerase and the mixture is incubated for a long period, say 12 hours.

All the three substances have been isolated. The P : N ratio corresponded in all the three to ADP. There is, however, a difference between the three in the behaviour of the phosphate groups. In ADP I only one phosphate group hydrolyses easily (7 minutes at 100° in *N* HCl), the second is hydrolysed only in 120 minutes. In ADP III both phosphates hydrolyse easily, while ADP II shows an intermediate behaviour, its one phosphate hydrolysing readily, the second in 90 minutes.

The three ADP:s react differently with isomerase and actomyosin. The whole of the labile phosphate (half of the total P) of ADP I hydrolyses off readily. ADP III is not attacked by

isomerase and actomyosin at all. ADP II shows an intermediate behaviour: half of its one labile phosphate ($^1/_4$ of the total phosphate) is hydrolysed off. This admits the conclusion that ADP II is a mixture of ADP I and ADP III.

As to the mechanism of the formation of the two different ADP:s it seems probable that ATP itself is not a homogeneous substance but is itself a mixture of two different substances, only one of which is attacked by myosin. If we suppose that there are two ATP:s, ATP I and ATP II, then one phosphate group of the one (ATP I) would have to be hydrolysed readily by myosin while the other (ATP II) would be hydrolysed only very slowly. In this case ADP I would be the product of hydrolysis of ATP I and the action of "isomerase" would be to convert ATP II into ATP I. A few observations indicate that ATP I corresponds to LOHMANN's formula of ATP, while ATP II, corresponds to BARRENSCHEN's formula.

After this chapter had been written BANGA found in several experiments that, contrary to the above experience, the whole of one phosphate of ATP was readily split off by myosin. The second phosphate was readily detached after addition of actin and isomerase. This newer experience, which is at variance with the older one, shows that there are still unknown factors. Whether the cause of the variation of results was due to a difference of the myosin or of the ATP preparation, remains to be shown.

Adenosine-diphosphatase.

I. BANGA observed that the isomerised ADP is not hydrolysed by myosin. A very slow reaction can be obtained, with a pH optimum at pH 7, in the presence of Ca. The hydrolysis of the isomerised ADP by myosin could be greatly speeded up by the addition of a third protein, extracted from acetone-dried muscle. All the properties of this third protein agree with the properties of actin so that we can say that isomerised ADP can be attacked by actomyosin and not by myosin.

The splitting of ATP and ADP by actomyosin depends in a different way on the KCl concentration (Fig. 47). While the ATP splitting has a flat maximum, the hydrolysis of ADP is limited to a fairly narrow range of KCl concentrations. Above 0.1 *M* the reaction is greatly inhibited. The KCl, evidently, dissociates

the actomyosin (isomerised ADP being present) into actin and myosin which, in themselves, are both inactive.

The pH optimum of the splitting of ADP by actomyosin was found to be 8.2. This reaction was inhibited by Ca ions and was enhanced by phosphate. Thus the phosphate, liberated by the

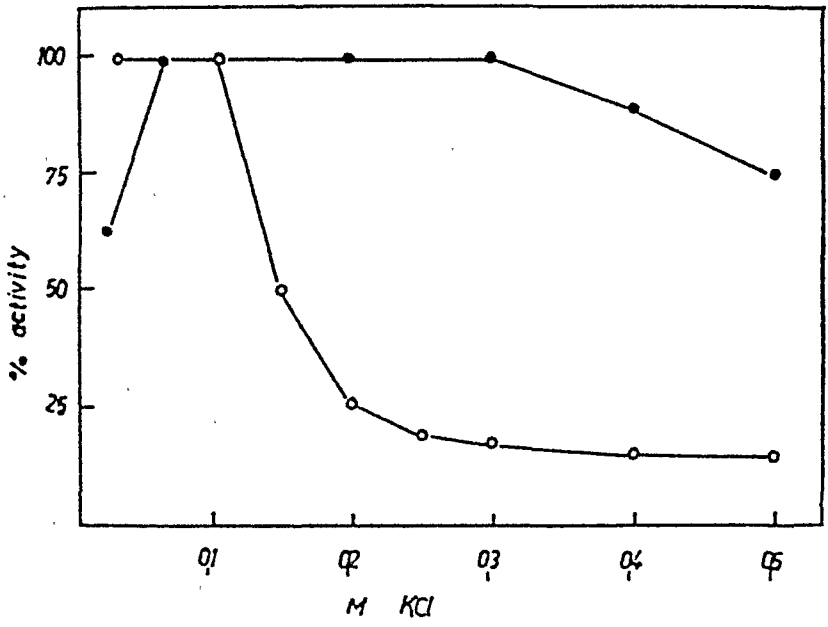


Fig. 47. Effect of KCl concentration on splitting of ATP and ADP. 100 % is the maximal amount of P split off under optimal conditions. ○ — ○ — P liberated from ADP, ● — ● — P liberated from ATP, 2 mg myosin, 3 mg of K ATP or ADP, KCl of varying concentrations. Total volume 3 ml. Incubated for 5 min. at 38°. In the case of ADP, isomerase and third protein were added, too.

splitting of ATP, may catalyse the further splitting of the resulting ADP.

In order to split off phosphate from ADP, we need thus three proteins: myosin, actin and isomerase. This three-protein system is activated by Mg. If the concentration of Mg is varied, a flat maximum is found at 0.001 *M* $MgCl_2$. On the other hand, as found by LAKI and BANGA, the system can be inhibited by pyrophosphate, which binds the Mg. The Mg, in this case, evidently acts on the isomerase. The activity of isomerase is accelerated by Mg by several hundred per cent. The activity of the three-protein system is activated at a lesser degree because the isomerisation is not the limiting factor of the reaction.

The ATP-creatine phosphopherase.

"It has been shown by K. LOHMANN[1] that in muscle the easily hydrolysable phosphate of ATP is taken over by creatine. In dialysed muscle-extract therefore the reaction taking place, can be expressed by the equation:

ATP + 2 creatine = adenylic acid + 2 creatine-phosphoric acid.

This reaction was investigated by H. LEHMANN,[2] who demonstrated its reversibility, but the equilibrium constant K calculated for a trimolecular reaction was not constant." (BANGA 134.)

BANGA showed, that this "reaction takes place in two distinct steps and that two different proteins are necessary for these reactions". The first reaction is:

ATP + creatine = ADP + creatine-phosphoric acid.

The enzyme catalysing this reaction was called the ATP-creatine phosphopherase and was purified to a degree at which no secondary reactions occurred. If the kinetics of this reaction were examined at varied concentrations of the reactants, the equilibrium-constant, calculated for a bimolecular reaction, was found to be constant. (Tab. X.)

Table X.

Experimental technique: 1 ml of veronal-acetate buffer solution of pH 8.55 + 0.1 ml = 60 γ ATP-phosphopherase + varying quantities of ATP and of creatine. Volume: 1.6 ml Incubation at 38°, until state of equilibrium had been reached.

M creatine added	*M* ATP added	*M* P found	$K = \frac{Cr.P.ADP}{ATP.Cr.}$
0.02390	0.00222	0.00104	0.040
0.02390	0.00444	0.00158	0.042
0.02390	0.00890	0.00232	0.038
0.02390	0.01331	0.00292	0.039
0.01030	0.00236	0.00078	0.040
0.01531	0.00236	0.00090	0.038
0.02040	0.00236	0.00104	0.040
0.02542	0.00236	0.00108	0.038

The second step of the reaction:

ADP + creatine = AMP + creatine-phosphoric acid.

This step of the reaction is catalysed by a protein which was called the ADP-creatine phosphopherase (BANGA 134).

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[1] K. LOHMANN. Biochem. Z. *271*, 264, 1934.
[2] H. LEHMANN. Biochem. Z. *281*, 271, 1935.

1 γ of the purified ATP-creatine phosphopherase transferred at pH 8.55 in one minute 2 γ P from ATP on to creatine. If the MW of the enzyme would be 70,000 then one molecule of the enzyme would react upon 4,400 substrate molecules per minute.

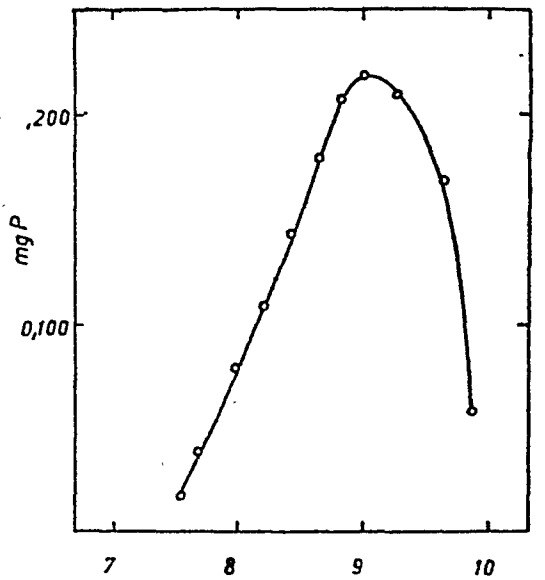

Fig. 48.

The pH dependence (borate buffer) is given in the curve of Fig. 48. The optimum lies at pH 9.05.

BANGA found that the value of the equilibrium-constant K depended to a great extent on the pH and reached its maximum at the pH optimum of the reaction (Tab. XI). On storage the pH optimum changed.

Table XI.

Experimental technique: 1 ml of borate buffer solution of varying pH. 0.0195 *M* creatine, 0.00890 *M* ATP, 0.1 ml = 60 γ of ATP-phosphopherase. Volume 1.6 ml. Incubation at 38° until the state of equilibrium had been reached.

pH	Measured *M* creatine P	$P K = \frac{Cr.P.ADP}{ATP.Cr.}$
7.50	0.00059	0.0023
7.60	0.00085	0.0049
7.80	0.00172	0.0239
8.20	0.00289	0.0925
8.50	0.00383	0.1910
9.05	0.00459	0.3651
9.50	0.00344	0.1382
9.70	0.00094	0.0061

As to details of standardisation and isolation see BANGA's paper (134).

PART VI.

Methods.

The preparation and crystallisation of myosin.

General remarks.[1] Myosin crystallises as a K-complex at pH 6.5, the isoelectric point of the myosin-K which is formed in the presence of 0.02—0.04 *M* KCl.[2] The crystallisation is promoted by the coaxial orientation of the micels, which orientation can be produced by strong stirring.

Myosin is very sensitive to the action of heavy metals: even as small quantities of Cu, as can be found in common distilled water, will suffice, under certain conditions, to denature it. For this reason, in all the work reported in this book, distilled water was used redistilled from glass vessels and only reagents of high purity were employed. Metal mincers, which give off metal easily, were discarded.

Myosin aggregates, at neutral reaction, in the presence of KCl, if the concentration of the salt is lower than 0.4 *M*. So if we want to extract myosin, the final concentration of the salt-solution should have at least this concentration.

It will not be solely the solubility of myosin which decides the result of its extraction. The result depends on the interaction of actin, myosin, ATP, salts and other substances. Owing to the joint action of the salts, used for extraction, and the ATP present, the actomyosin of muscle is dissociated into actin and myosin; the actin is retained, the myosin is dissolved. In absence of ATP the insoluble actomyosin is formed. For this reason it is essential to use fresh muscle, rich in ATP and treat the muscle

[1] By this method recrystallised myosin was prepared daily for a long time as a routine by a technical assistant with only exceptional failures.

[2] Probably other alkali-metals behave similarly but have not been tested yet.

carefully, giving little chance for the splitting of the ATP. The action of ATP is greatly enhanced also by the Mg and the isomerase present in muscle.

This separation of actin and myosin, however, is not very sharp and along with myosin some actin is dissolved. The quantity of actin increases on prolonged extraction, but its dissolution follows a different curve than that of myosin. The subsequent separation of this dissolved actin always entails a great loss of myosin. For this reason, if we want to prepare actin-free myosin, it is essential to start with an extract as inactive as possible. Higher pH increases the solubility of actin, lower pH decreases the solubility of myosin. As shown by GUBA and STRAUB (130) the optimal pH is 6.5.[1]

Actin is brought into solution in different muscles and in different animal species with different ease (F. GUBA 129). For the preparation of actin-free myosin an animal species has to be chosen in which myosin is dissolved easily and actin retained strongly. The striated muscle of the rabbit is an especially favourable material. For this reason this material was used with preference and all the data in this book relate to it, if not expressly stated otherwise.

Myosin is labile and sensitive to heat. So we have to work fast and at a temperature as low as possible. Unfortunately not all steps can be effected at 0° because actomyosin is not precipitated by ATP at this temperature. The actomyosin, precipitated by ATP at room temperature, dissolves even at 0°. So it is not possible to work throughout at this temperature. Fortunately ATP stabilises myosin against heat. This stabilising affect was noted by ENGELHARDT and LJUBIMOWA, corraborated by J. NEEDHAM and his collaborators and D. M. NEEDHAM.

Myosin cannot be freed from actin by crystallisation since actomyosin behaves as an individual substance and even a 2.5 % actomyosin can be crystallised as such (F. GUBA, oral comm.).

The principle of the method of preparation of myosin is the following: the muscle is extracted with the KCl-phosphate solution of pH 6.5 of GUBA and STRAUB. The extract is diluted and the greatest part of the actin is precipitated by the ATP present in the form of 1.5 % actomyosin.

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[1] Since glycogen also precipitates myosin, it is advisable to use rabbits which were fasting for 1—2 days.

The ionic strength, at this stage, corresponds to a 0.1 *M* KCl. At this salt concentration ATP precipitates a 1.5 % actomyosin. Thus one part of actin will take down 66 parts of myosin. (At higher salt concentrations ATP precipitates more active actomyosin, but the precipitation is less complete; above 0.2 *M* KCl no actomyosin is precipitated at all.) Only the excess of myosin is left in solution, which the actin is unable to take down. Thus, if the primary extract contains 1.5 % actomyosin, no actin-free myosin can be obtained by the present method at all.

The myosin is brought to crystallisation by further dilution.[1] In order to liberate it from the last traces of actin present and some inactive matter (glycogen?), it is dissolved in 0.04 *M* KCl at an alkaline reaction. After the insoluble matter has been separated the myosin is precipitated by neutralization. It separates in crystals and may be recrystallised a second time by dissolving it in 0.6 *M* KCl and diluting the fluid to 0.04 *M*.

Yield. Muscle contains 8 % myosin. About $^1/_3$ of this quantity is extracted by the KCl-Phosphate. Half of the extracted myosin is left behind in the fluid after the first precipitation. Another $^1/_2$ is lost on recrystallisation. About 10 % of the myosin, originally present in muscle, will be obtained in the final recrystallised condition.

The method of preparation. The animal is killed, quickly skinned, eviscerated and dipped into ice-water. After a few minutes the muscles are cut out and packed into ice. Then they are minced in a cooled meat mincer with holes of 2 mm diameter.

Every 100 g of the minced muscle are suspended in 300 ml of an ice-cold solution containing 0.3 *M* KCl and 0.15 *M* K-phosphate of pH 6.5.[2] The muscle is extracted for ten minutes at 0° under constant stirring. Stirring by hand with a glass rod is convenient. (Too energetic mechanical stirring should be avoided.) Then the suspension is diluted with water of room temperature (22°), four volumes of water being used for every volume of KCl-phosphate. The suspension is rapidly strained through a

[1] The extract has to be diluted to 0.04 *M* KCl concentration. If we start with 1 l KCl-phosphate, the extract has to be diluted to 12 l. This great dilution entails a considerable loss in myosin. At 0.02 *M* KCl the precipitation of myosin is more complete, but the higher dilution does not increase the yield.

The most convenient method seems to be the electrodialysis. With this method the extract, diluted to 5 l, could be brought down to an ionic strength, corresponding to 0.02 *M* KCl, without dilution. Unfortunately, owing to lack of apparatus, this method could not be tested.

[2] In order to save water the muscle may be extracted with half this amount of KCl-phosphate, containing the salts in 20 % higher concentration. For the subsequent dilution 5 volumes of water have to be used instead of 4 vol.

cloth, the fluid gently stirred by a mechanical stirrer. After one or two hours a flocculent precipitate is suddenly formed. This happens because the ATP is split to such an extent, that instead of dissolution, it causes the actomyosin present to precipitate. If the precipitation takes place without stirring, a very fine colloidal precipitate is formed which cannot be separated on the centrifuge. If the stirring is too violent, the precipitate is disaggregated.

The precipitate, thus formed, is separated by rapid centrifugation at room temperature and the opalescent fluid is diluted with 1.5 vols of ice-cold water. This water is run in slowly, in about 10 minutes, under constant energetic stirring. The myosin separates in the form of fine, needle-shaped crystals.

The fluid is allowed to stand for an hour or two at 0° C, then decanted and the myosin separated on the centrifuge at 0°.

The crystalline precipitate is washed by suspending it in 0.02 *M* KCl and centrifuging it.

The crystalline myosin precipitate is dissolved in a 0.02 *M* K_2CO_3 containing 0.01 % phenolphtalein. The carbonate solution is added till the fluid retains a faint rose colour (pH 8.3). Then we add, for every g of myosin present, 4 ml of 2 *M* KCl and dilute with water adding 50 ml for every ml of KCl solution used. This water is of room-temperature (20° C) and contains 0.001 % phenolphtalein and sufficient K_2CO_3 to give it a faint rose colour. The water is added under strong stirring. A voluminous, loose precipitate is formed which is separated on the centrifuge. The faint rose coloured opalescent fluid is poured off and cooled. The precipitate is treated once more in the above way, *i. e.* if its colour has faded out, it is restored by adding K_2CO_3, then we add KCl and finally water, and centrifuge, the only difference being that this second time we add only half as much KCl and water as the first time. The precipitate is discarded and the fluids united. After this the preparation is continued at 0° C.

The fluid is stirred energetically and 1 % acetic acid is run in very slowly till the fluid reached pH 7. The myosin precipitates in form of somewhat irregular needles which are separated on the centrifuge.

The precipitate is dissolved by adding 2 *M* KCl to it in small quantities. The fluid is carefully homogenised after each addition. KCl is added till the concentration of the KCl reaches 0.6 *M*. Then we dilute further with 0.6 *M* KCl till the fluid loses its very high viscosity and contains about 3 % myosin.

The myosin solution is stirred very energetically and water is run in very slowly till the KCl concentration is brought down to 0.04 *M*. The addition of this amount of water should take about one hour. The myosin separates in the form of needle-shaped crystals. This myosin contains no actin or only traces of it (0—0.2 %).

The preparation of actin.

STRAUB's *method*. The method of preparation of actin consists of five steps: mincing, extraction of myosin and soluble proteins, alkali-treatment, acetone-treatment, and extraction of actin.

The muscles of the freshly killed rabbit are rapidly excised, cooled immediately by packing them into ice. Then they are minced first by a cooled meat chopper (diameter of holes 2 mm) and then by a cold LATAPIE mincer (diameter of holes 1 mm). 300 ml of an alkaline KCl solution[1] are added to every 100 g of the mince and stirred mechanically at 0° for 20 minutes. The mixture is then centrifuged and the supernatant fluid, which contains the greater part of the myosin and some actin, is discarded. The residue is left to stand at 0° for 24 hours. At the end of this period it is weighed and mixed with 5 volumes of its weight of distilled water of room temperature. After standing for 1 hour the mixture is centrifuged. The washing of the residue with distilled water is repeated with the same amount of water as before, again standing for 1 hour. After this second washing the muscle residue is treated with 4 volumes of acetone of room temperature. After 20 minutes standing the acetone is removed by pressing it out through a cloth. The residue is now mixed with a fresh lot of acetone ($^1/_4$ of the former volume) and left to stand again at room temperature for 20 minutes. The acetone is pressed out and the residue spread over filter paper and left to dry.

The aceton-dried muscle powder (after 10—15 hours of drying) is extracted with 20 volumes of CO_2-free water of room temperature. The muscle powder is mixed with the water and left to stand for 10—15 minutes. At the end of this period the resulting pulp is poured into a Buchner funnel and the solution is sucked

[1] This is prepared by mixing 800 ml 0.1 *M* potassium borate with 200 ml of 2 *M* KCl. — The potassium borate solution is made by dissolving 12.4 g boric acid in 100 ml *M* KOH, then making the solution up to 1 l with dist. water.

off. It contains the actin in its inactive form. The protein content varied between 3—6 mg/ml. The purity of the actin in such a solution is mostly maximal (1.0) but rarely below 0.7.[1] If the purity of the preparation is not maximal it cannot be raised any further. The extract contains mostly about 5 mg actin per ml. The yield might be increased by stirring and a more thorough extraction of the dry muscle. Yet in such cases the resulting solution will be opalescent, and the actin will become activated. Therefore higher yields have to be sacrificed for the advantage of obtaining inactive actin in clear solution.

Modified method. The author, in collaboration with F. GUBA, adjusted STRAUB's method to the use of the muscle residue of myosin preparations. This modified method is not more involved than the original one. The yields are equal.

The muscle is minced, extracted with acid KCl-phosphate, diluted, pressed out as described in the myosin preparation. The muscle-residue is weighed and suspended in 5 volumes of 0.4 % $NaHCO_3$ solution. The suspension is stirred for thirty minutes, then strained, the muscle pressed out and minced on a LATAPIE mincer.[2]

Every 100 g of the mince are suspended in 100 ml of a solution containing 0.05 *M* $NaHCO_3$ and 0.05 *M* Na_2CO_3. The suspension is allowed to stand for ten minutes and then diluted with 10 volumes of water, stirred for ten minutes and then centrifuged. The sediment is mixed with equal volumes of acetone, strained and gently pressed out. The residue is suspended in its equal volume of acetone, allowed to stand for ten minutes, strained and than pressed out. This acetone treatment is repeated once more, the muscle spread on filter paper and dried, and is extracted as described in the first method.[3]

F. GUBA (oral comm.) simplified this method. In his modification the muscle, after treatment with $NaHCO_3$ is minced, as described before, and then it is put over night into the refrigerator of —12°. (The muscle may be kept here for any length of time). If thawed, treated with acetone, dried, ground and extracted, it will yield up its actin as in the other methods.

[1] The purity of actin is maximal (1.0) if 1 mg of it gives, with 5 mg pure myosin, a 100 % active actomyosin.

[2] If such a mincer is not available repeated mincing on the ordinary mincer with 2 mm holes may do.

[3] The extract should not colour phenolphthaleine. If it does so mincing was not fine enough and the removal of alkali was incomplete. Alkali destroys extracted actin.

Isoelectric precipitation. The preparation may be finished with an isoelectric precipitation which allows to prepare solutions of high purity and high actin concentration. G-actin precipitates to a small volume, while F-actin gives very bulky precipitates. The solutions, obtained by the described methods, contains actin in its G-form.

A pH 4.7 acetate buffer was prepared by mixing *M* Na acetate and *M* acetic acid. The buffer was cooled in ice. 5 ml of the ice-cold buffer were added to every 100 ml of the ice cooled extract under strong stirring (more buffer denatures actin). The fluid was quickly centrifuged in the cold. It needed only a very short spinning. The clear supernatant fluid was poured off and the residue dissolved in a small quantity of 0.1 *M* Na_2CO_3, excess being avoided. A limpid solution was obtained which contained the actin partly in its G- and partly in its F-form.

G-actin precipitates quantitatively if present in higher concentration. From dilute solution the precipitation is sluggish and incomplete. Actin, precipitated isoelectrically, showed maximal activity and could not be purified any further by other procedures (alcohol precipitation, salting out). It behaved as a homogeneous substance and its total protein was precipitated by small Ca-concentrations. This makes it probable that such an actin does not contain any significant amount of impurities.

DRF.

Whether a substance has a DRF or not is relative. So, for instance, benzol has, at very high velocity gradients, a DRF. A slight anisodiametry is sufficient to cause DRF under these circumstances. But even molecules, devoid of any such asymmetry may exhibit a DRF being deformed by hydrodynamic forces. In the problems discussed in this book, such slight or enforced molecular asymmetries were of no avail. We were interested only in extreme dimensional asymmetries as exhibited by the typically highly polymer fibrous colloids. Such particles are oriented even at very low velocity gradients and are not desoriented easily by thermal agitation. High velocity gradients are not only not needed but have to be avoided, or else we would not obtain an answer to our special question: whether a certain substance has a very high dimensional asymmetry or not.

Thus by saying that a substance has a strong, a weak DRF or

has no DRF at all, we mean only DRF to be observed by direct observation at low velocity gradients. Such observations do not necessitate any involved apparatus.

The simplest method for the study of DRF consists of pouring the solution into a test tube and sucking it up and squirting it out with a pipette with a narrow end. The jet of fluid is observed between crossed Nicols. If the solution shows a "weak" DRF, then the sides of the jet will show a faint luminosity. As the DRF increases, the light will become stronger, the lighting area bigger. In case of strong DRF the whole fluid will become luminous on agitation. In extreme cases the DRF will be permanent and persist even after we stopped stirring. Naturally, DRF depends on concentration and sufficiently strong solutions, say 0.2 %, should be employed.

As polarisers and analysers the ZEISS-BERNAUER filters were employed. They are cheap, easily mounted and have a wide field of vision. M. GERENDÁS (123) has described a simple arrangement. He also described a cuvette for the observation of the DRF.

v. MURALT and EDSALL have described the method of quantitative work. If completed with photoelectric registration their apparatus may give precise data. Till, however, we are informed about the relation of DRF and molecular structure, exact measurement will probably not give much new information.

The estimation of myosin.

Myosin can be estimated by gravimetry or by viscosimetry. It will depend on conditions which of the two methods is preferable. If we have a myosin solution, free of other proteins, then we can simply precipitate the protein and estimate its quantity gravimetrically. The most convenient precipitating agent is alcohol. The solution is diluted till the KCl concentration falls below 0.2 *M*, an equal volume of alcohol is added and the mixture heated for ten minutes to 70°. The precipitate is centrifuged off and washed with 50 % alcohol and dried at 105°. If this is done in a weighed centrifuge-tube, quick and reliable results may be obtained. Trichloroacetic acid is not convenient as a precipitating agent because the precipitate encloses rather big quantities of it and washing entails loss.

From a mixture of proteins myosin can be precipitated in the presence of small (0.1 *M* KCl) salt concentrations isoelectrically

at pH 5.3, according to BALENOVIĆ and STRAUB (117). This method can also be used for the estimation of actomyosin.

The quantity of myosin can also be estimated in the viscosimeter. The viscosity of myosin depends on pH and salteoncentration, so both must be well defined. The viscosity can be evaluated by means of an empirical curve, like that of Fig. 5. This curve was obtained in 0.6 *M* KCl at pH 7, in presence of the veronal-acetate-KCl proposed by BALENOVIĆ and STRAUB (117).

This solution is prepared in the following way: 21 ml of *N* HCl were added to 200 ml of the veronal-acetate mixture of MICHAELIS. This mixture was prepared from K- instead of Na-salts. To this mixture were added 271 ml of 2 *M* KCl and the volume filled with water to 1 liter. The final K concentration was 0.6 *M*.

The solution should not contain more than 2 mg myosin per ml. It is the most convenient to work with quantities of about 1 mg per ml. In all experiments the temperature was kept constant at 0°. Modified OSTWALD viscosimeters were used, as specified by STRAUB (116).[1]

The estimation of the myosin and actin content of actomyosin. Activity.

By "natural actomyosin" I shall mean here the actomyosin extracted from muscle as such, as contrasted with the actomyosin prepared from myosin and actin. If the natural actomyosin is dissolved in 0.6 *M* KCl of pH 7 and 0.035 % ATP is added, the actomyosin dissociates. The viscosity will now be the viscosity of myosin plus the viscosity of actin. If we measure the viscosity before and after the addition of ATP, the difference will be the "activity".

The activity of myosin B in its relation to concentration is given in Fig. 49, quoted from BALENOVIĆ and STRAUB.

If we call the activity of myosin B "100 % activity", we may express the "activity" of any actomyosin numerically by comparing it with the activity of myosin B. We proceed as follows: we estimate the activity of the unknown actomyosin (the difference of specific viscosity before and after addition of ATP). Then we look up in the curve the quantity of myosin B which gives the same specific viscosity in presence of ATP, as the un-

[1] Capillary diameter 0.060 cm, length of capillary 210 mm, diameter of the cylindrical reservoir tube 1.65 cm, amount of outflowing fluid 1.2—1.7 ml.

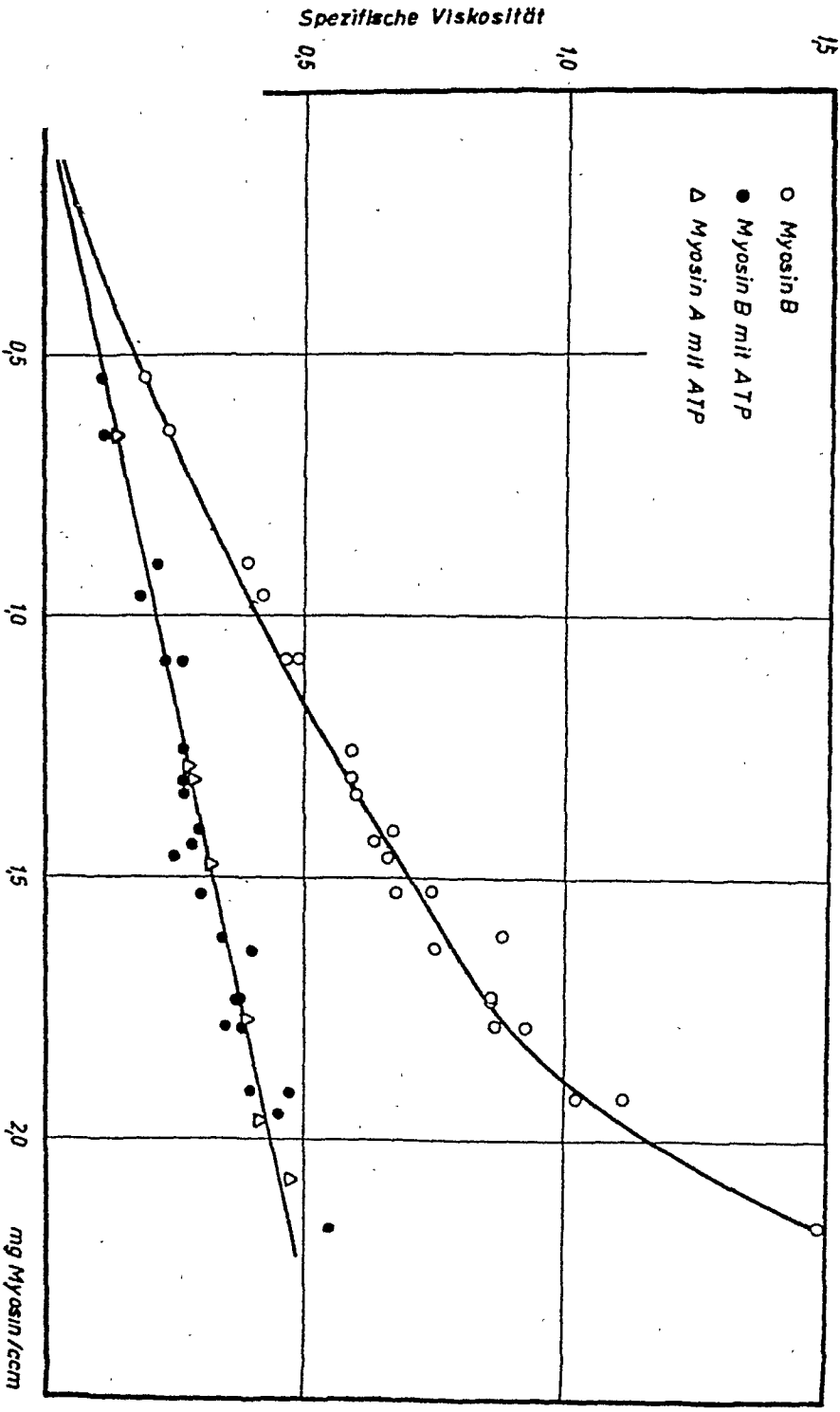
Spezifische Viskosität
0,5
1,0
1,5
○ Myosin B
● Myosin B mit ATP
△ Myosin A mit ATP
0,5
1,0
1,5
2,0
mg Myosin/ccm

Fig. 40.

known solution. The activity of the unknown solution divided by the activity of this corresponding myosin B concentration, multiplied with 100 gives, what we called the % activity. The relation of the actin content and % activity is given in Fig. 50,

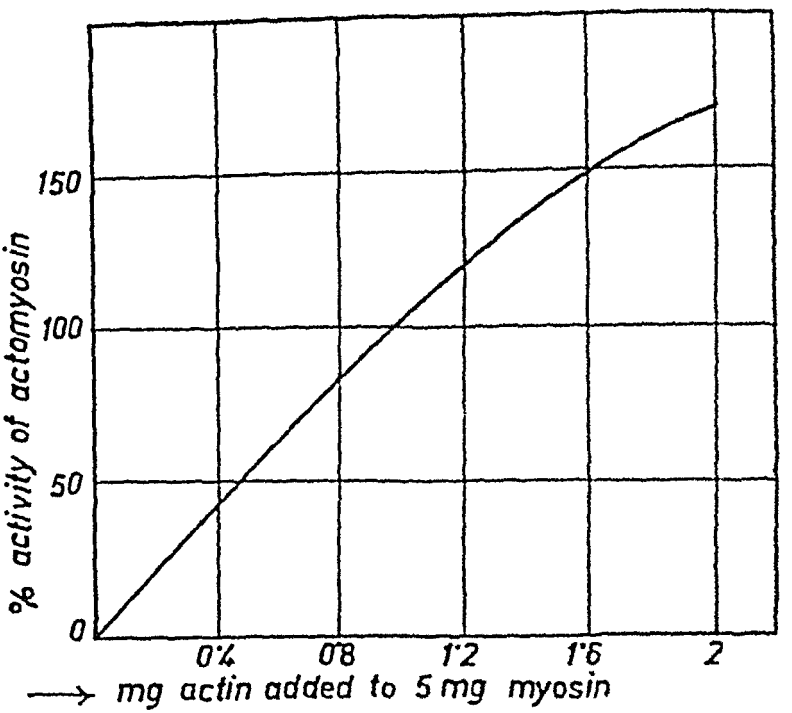

Fig. 50.

quoted from STRAUB. Once we know the % activity, the actin content may be read from this curve.

Only the F-actomyosin will answer these relations. The viscosity of G-actomyosin is identical with the viscosity of myosin. One is not likely to meet G-actomyosin, except under special circumstances, because the G—F transformation of actin is greatly catalysed by myosin and thus G-actomyosin is quickly transformed into F-actomyosin. Only in presence of high salt concentrations is this catalysis inhibited and G-actomyosin stable. To enforce the G—F transformation in this case we will have thus to lower the salt concentration or add ATP, in which latter case the actomyosin dissociates and the actin polymerises.

Actomyosin, prepared from actin and myosin, differs from the natural one in two respects. While the latter shows a constant

viscosity, the viscosity of the former drops in time and has to be stabilised by Mg (0.001 *M* $MgCl_2$). This actomyosin is also more thixotropic.

If the actin-content of an extremely inactive actomyosin has to be estimated, the effect of actin on viscosity and thus the "activity" can be magnified by working, instead of at pH 7 and 0.6 *M* KCl, at a lower pH and lower KCl concentration (see STRAUB 112).

The estimation of actin.

No chemical property of actin is known at present on which a method of determination could be based. The only basis of determination can thus be its special reaction with myosin which can be followed quantitatively in the viscosimeter.

The quantity of actin in solution can be estimated in the following way: an actomyosin-solution as inactive as possible is prepared by extracting muscle for ten minutes with the acid KCl-phosphate mixtures. After the ATP present is decomposed,[1] the % acticity and thus the actin-content are estimated. After the addition of a certain amount of our actin solution, again the % activity and the actin-content are estimated. The difference will be equal to the added actin.

Naturally, in this way only F-actin can be estimated. If we want to estimate the actin content of a solution of G-actin we have to activate it, by adding 0.001 *M* Mg and 0.1 *M* KCl and letting it stand at room temperature (22°) for a quarter of an hour. We can find the G- and F-actin content of a mixture of both substances by measuring the actin content before and after activation.

If the actin content of muscle has to be estimated this can be done, according to BALENOVIĆ and STRAUB (117), by estimating the maximum quantity of myosin which can be activated to 100 % by a given amount of muscle, after this muscle has been suspended in this myosin solution for 24 hours. The muscle should be finely divided. We may also measure the extent to which a great excess of myosin is activated by a given amount of muscle. Owing to the thixotropy and the instability, mentioned before, we must not employ pure myosin in these experiments, but an actomyosin of low activity, extracted from muscle.

[1] ATP is decomposed on storage over night at 0°.

If the actin-content of a solution is fairly high (0.2 % or more) and the actin is present as G-actin, then it can be precipitated isoelectrically at pH 4.7. From a more diluted solution precipitation will be incomplete and may be promoted by the addition of alcohol. The precipitation of F-actin at pH 4.7 can be promoted by a short strong shake which breaks up the thixotropic structure. The precipitated actin can be washed with alcohol, dried and weighed.

The preparation of ADP.
(Quoted from Banga 135.)

"100 mg crystallised myosin and 1,400 mg of ATP, in the form of neutral K salt, were dissolved in 300 ml 0.1 *M* KCl. Incubation at 38° until samples taken showed that one P had already been split off. The protein was then removed by adding 30 ml of 20 % trichloroacetic acid. Thereafter 3 g $MgCl_2$ were added and NH_4OH until the solution began to turn red in presence of phenolphtalein. The resulting mixture was stored over night at 0° during which period the inorganic P had separated quantitatively in the form of $Mg(NH_4)PO_4$. In order to precipitate the neutral Ba salt of ADP, 3 g of $BaCl_2$ and 1/5 volume of alcohol were added to the liquid. The precipitate was washed first with 50 % alcohol, then with absolute alcohol and dried. The yield was 65 % of the theoretical value.

In order to obtain the ADP free of Mg, the Ba salt was dissolved in 5 % acetic acid and precipitated by Hg acetate. The salt was dissolved in 0.5 *N* HCl and the Hg removed as HgS. H_2S was removed by ventilation. By adding $BaCl_2$ and alcohol to the resulting liquid at neutral reaction, the ADP was precipitated as Ba salt.

Analysis of P: 100 mg Ba salt of ADP were dissolved in presence of HCl in 8 ml of H_2O and K_2SO_4 was added (one molecule of K_2SO_4 to every molecule of Ba salt). Thereafter it was neutralised with KOH and the volume brought up to 10 ml with water and centrifuged. 0.1 ml of this solution contained 0.002 mg of inorganic P, 0.046 mg readily hydrolysable P and a total P of 0.093 mg. The proportion of the readily hydrolysable P to the stable one was found to be 1 : 1."

This method was worked out at a time when the existence of the three different ADP:s was not yet recognised. If we want to prepare ADP I we have to add isomerase, along with myosin, to

the ATP. The Ba salt of ADP I is very insoluble and will precipitate even without the addition of alcohol (BANGA unpublished). For the preparation of ADP II we do not add isomerase but stop the reaction when about 66 % of the first phosphate is liberated. In order to obtain ADP III we incubate for 12 hours. The Ba salt of this ADP is relatively soluble and an excess of alcohol has to be added to precipitate it.

Estimation of ATP.

If the minced muscle is extracted for ten minutes with 0.6 *M* KCl and centrifuged, a fluid is obtained which contains about 1—1.2 % of actomyosin with a 0.5—1 % actin content. If the extract is stored over night at 0° the ATP is split. If the extract is diluted now with 4 vol. of water,[1] containing 0.001 *M* $MgCl_2$, a slightly turbid fluid is obtained which contains about 0.1 *M* KCl and the actomyosin in the form of a stable suspension. If ATP is added now, according to its concentration, a turbidity or a clearing up will be seen.

If an unknown ATP solution is added to the extract and its effect is compared with the effect of a known ATP solution, conclusions can be drawn on the ATP content of the unknown solution, provided it did not materially change the salt concentration.

ADP may be estimated in the presence of ATP by repeating this estimation in the absence and presence of isomerase. In this case, naturally, we have to work, with a pure, isomerase-free actomyosin, instead of a muscle extract. As isomerase we may use a watery extract of muscle, adding Mg in excess (0.002 *M*) to all tubes. 10 % watery extract added to the reaction-mixture will be sufficient to provide maximum isomerase effect. To remove salts the watery extract may be dialysed.

This method may help to distinguish between ATP or ADP isomerase. We used this method where this had to be done. Otherwise we preferred to estimate the ATP chemically, estimating the quantity of free, labile and stable phosphate.

The method may be used also for kinetic studies on the splitting of ATP (139).

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[1] The water should be added suddenly. If it is run in slowly the actomyosin flocculates.

PART VII.

Considerations.

A mechanical model of contraction.

Any mechanical model of muscular contraction, hitherto proposed, had to make clear:

1. Why the DR is diminished or lost on contraction.
2. Why the muscle becomes thicker while becoming shorter.
3. How it is that the muscle exerts its biggest force when stretched?
4. Why body muscles are striated and why smooth muscle is not.

The theories and models hitherto proposed answered only the one or the other of these questions, though a model or theory if it really meets facts, has to give a complete and coherent explanation of all properties.

To the above demands we must add now the following:

5. The model should explain why two linear colloids are necessary to make a contractile system.
6. Why only the one of these two colloids is made in such a way that it is easily discharged, precipitated and dehydrated by ions, while the other is inactive?
7. How can a highly contractile system, capable of extreme shrinking, arise from the union of two substances the one of which is inactive?
8. Why is this latter, the inactive colloid, built in such a way that it is capable of existing in two forms, in a linear and a globular form, which can easily be transformed into each other?
9. Why only the linear form is capable of building a contractile system and not the globular one.
10. Why at the end of contraction this colloid is found in the globular form. How is it broken up during contraction into globules?

11. Why the two colloids are attached to each other in a loose dissociable form.

12. How the contracted system yields a relaxed system on dissociation.

13. How can the one of these substance catalyse the polymerisation of the other?

14. The shrinking of hydrophil colloids mostly consists of two parts: 1. the discharge of the colloid and subsequent loss of hydrate water. This part of the shrinking is not extensive, since the quantity of hydrate water is small, but it may take place very fast and involve big changes in free energy. 2. Removal of the intermicellar water. This part may be very extensive but entails no major changes in free energy and is useless as a source of work; it is mostly rather slow.

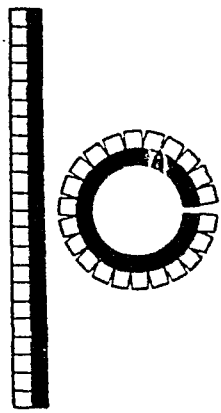

Fig. 51.

The contraction of actomyosin is fast and extensive, and so is muscular contraction which can be looked upon as a one-dimensional shrinking. A new model has to explain, how an extensive shrinking can not only be fast but do work through its whole extent.

The way to the establishment of a model, which answers all these questions, was opened by accidental observations made on actomyosin threads. If our actomyosin thread, suspended in saline is homogeneous and the ATP is added in such a way that it reaches the thread from all sides, then the thread simply contracts. But if the ATP is introduced on one side and reaches the thread asymmetrically, then the thread, before contracting, curls up, as shown in Fig. 51. This bending is caused by the one-sided shrinking of the thread. In the Fig. this shrinking side is marked with black. A simple geometrical consideration will show that the thread has to curl up into a circle if one of its sides becomes by $\pi \cdot d$ shorter, d being the diameter of the thread. If we take, for instance, a thread, 100 long and 1 wide, this thread will curl up to a circle if one of its sides shrinks by three units. By this curling up the effective linear length has shortened by 2/3.

It is immaterial what the cause of the asymmetry is. If the thread shrinks asymmetrically, it has to bend. If the threads are not prepared under special precautions they very often have an asymmetry in their structure and bend or curl up on addition of

ATP; especially thin threads often do so. This bending of asymmetrically shrinking or expanding systems is also a very unpleasant experience of every-day life being the cause of the bending of wooden boards.

The actomyosin micel is such an asymmetrical system, of which only one half, the myosin, is capable of shrinking, being discharged by ions. Fig. 51 can be taken to represent the actomyosin micel, the myosin moiety being marked with black. (The really high degree of asymmetry seems to be introduced into this system by the ATP).

If the shrinking of the myosin moiety leads to a bending of the system, the bending of the system will lead to a stretching of the actin, which comes to lie on the outer circle. Actin thus has to resist bending, except if it is capable of breaking up, as shown in the Fig. 51.

In this mechanism a slight shrinking of myosin will cause a bending and herewith an extensive shortening of the whole system. This mechanism is thus an amplifier, by which the first slight, but energetically important part of the shrinking is amplified to a very extensive shortening. If our system consists of single actomyosin micels, distributed at random, then the contraction of the system will be isodimensional and its maximum will be reached when the single micels have curled up to a circle and the whole system has shortened by $^2/_3$. $^2/_3$ has been found in the experiment to be the limit of contraction of myosin threads, though this limit might have also a different explanation.

If the myosin micels in the thread are oriented coaxially, then the thread must become not only shorter, but also wider on contraction and has to loose its double refraction.

According to this model the biggest force will be exerted at the beginning of the contraction, when the system is still unbent. At this stage much bending will cause relatively little shortening, thus the product: way × force, will be the biggest.

If the contracted actomyosin dissociates, the actin and myosin particles straighten out. A system, built of one rod (myosin) and globules (G-actin), will not bend, thus not contract. The rod may catalyse the polymerisation of the globules by binding them and bringing them this way into proximity.

Steric relations. The non-existence of cross striation.

In the previous chapter a mechanical model of contraction has been developed, according to which the bending of the actin-myosin double-rod contributes to the shortening of the system and the active expulsion of intermicellar water. The question arises what is the actual structure and distribution of these rods in muscle. Muscle has two properties which may help us in answering these questions: its close packing and its mechanical strength.

The packing in the muscle-fibril is exceedingly close, indeed. Muscle contains about 8 % myosin and 3 % actin, both located in the fibril which occupies not more than about $^1/_3$ of the total volume of the muscle fibre. Accordingly the fibril contains 33 % actomyosin and no more than 67 % of water. A considerable part, at least the half of this water must be bound by the strongly hydrated proteins as hydrate-water. The volume relation of the hydrated protein to the intermicellar space is thus about $^2/_3 : ^1/_3$, only $^1/_3$ of the volume being occupied by water-filled intermicellar space. With such a close packing the particles must fit exceedingly well together.

Muscle has a considerable mechanical strength: it resists stretching or tearing. This resistance is not very different in the resting and in the contracted muscle. It can be explained only by supposing that both, the myosin and actin contribute to it and form continuous systems. As will be shown later, actomyosin in resting muscle is dissociated and we have actin and myosin side by side. If, all the same, muscle resists stretching, this can only be if actin and myosin form a continuous system in themselves and form a continuous actin- and myosin-system.

The actin and myosin, however, cannot form very long straight rods, because such long, straight rods would need very much space for bending, which is not available in the closely packed fibril. There is only one distribution which answers all requirements: if the one of the two proteins forms a long rod or thread while the other is wound around it in a spiral. This structure would resemble a screw or rather a worm-gear and would, on contraction, bend to something like a cork-screw.

It has been shown that actin has a strong tendency to form long threads while myosin has a strong tendency for lateral association. It has also been shown that, in muscle, actin binds its

multiple weight of myosin. This makes it probable that actin actually forms long threads in muscle and that, if actomyosin is formed, the elongated myosin-particles are attached to the actin

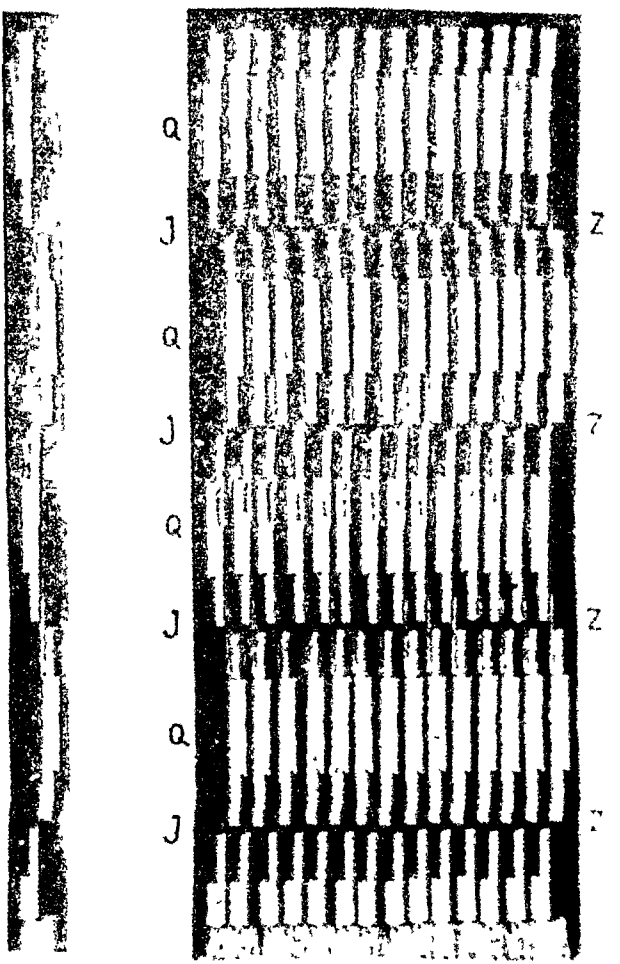

Fig. 52. Where the myosin stands to the right or left, its DR will add up with that of the actin, while at 90° to this the DR-s will substract. If we place a number of such models side by side in such a way that the spirals of a cross section are in the identical phase, the system will show a cross striation. (Fig. 52 B.) This cross striation, natually, will be an optical property only and the system having no segmental structure whatever.

particle with their one end, stand radially and stick to the neighbouring myosin micels with their long side.

If we assume that the muscle fibril is actually built of such

spirals we can make one more statement. Such a spiral structure is compatible with the close packing only if the screw-threads of the one spiral fit into the screw-groove of its neighbours. This would make that, in any cross section of the fibril all the spirals will be in an identical phase, that will say that all the myosin-spirals will stand at the same side of their actin-threads.

Such an actin-myosin-spiral is found in the model of fig. 52 A. On this model the continuous myosin-spiral is represented by single celluloid plates which can be looked upon as radial cross sections of the spiral. If we look at such a spiral from the side, its optical properties change periodically as the myosin is winding around.

On contraction these regularities are disturbed, the DR decreases, the striation becomes narrower. The whole system becomes shorter and wider and its contour-line must show a periodic indentation with a maximum in Z.

The properties of this system of spirals agree so closely with the properties of striated muscle that it becomes highly probable that the cross striation of body muscle is due to the spiral structure of the actin-myosin system. This assumption is accessible to experimental proof. If the cross striation of muscle is not due to a real segmentation but is only an optical property, then, on turning the muscle fibre or fibril round, the cross striation has to shift, and has to shift by one period if the fibre is turned by 180°.

M. GERENDÁS performed this experiment by drying a stretched frog-muscle, breaking it to pieces and sticking a fragment on a pin's point and rotating the pin in the micromanipulator under the microscopy. He could see a shift but was unable to state the relation between the extent of the shift and degree of turning.

The author proceeded in the following way:

The muscles of the insect *Dytiscus marginalis* were cut out, suspended in about 3 ml of 25 % glycerol, to which 1 small drop of saturated osmic acid and 2—3 glass pearls were added. The suspension was energetically shaken. After the muscles have fallen into fibrils, the mass was allowed to stand over night at 0° and then mixed with equal parts of 10 % gelatin. The gelatinous suspension was frozen on the freezing-microtome and cut into about 0.040 mm thick slices. The slices were dissolved in 3—4 ml of 10 % gelatin of 37° and the solution kept at 37° and sucked into 15 cm long capillary glass tubes of 0.3 mm outer and less than 0.1 mm inner diameter. The tubes were allowed to cool in ice-water in vertical position.

Then the tubes were fixed on to a microscopic slide by means of two stripes of paraffin-wax, as shown in Fig. 53. First a thin sheet of paraffin was put on by means of a brush. Then the wet tubes were placed on the slide and fixed in their position by going over them with the brush. Then the layer of paraffin was rendered thick by going over

Fig. 53.

it again and again with the brush dipped in melted paraffin. The paraffin serves not only to fix them, it serves also as a perfectly fitting axle-bearing which allows to turn the tubes without shaking. Since the shifts to be observed are of the dimension of 0.001 mm, it is essential that there should be no shaking whatever. The tubes could be turned by means of a small glass rod stuck to the end of the capillary at 90° by means of sealing-wax.

The middle-part of the tubes was covered with oil and a cover-glass and the microscopy focussed on the axis of the capillary. Then the capillary was drawn along the field of vision. What one wants to find is a short, straight piece of a fibril, which shows a strong cross striation and lies close to and parallel with the axis.

Attempts to measure the shift of the Z-lines relative to a fixed point were unsuccessful because the whole capillary often moves on turning to one side. The shift of the Z-lines could be observed relative to the end of the fibril. The striation, on turning, was seen to move out of the fibril at one end and move into the fibril on the other. The shift, observed on turning by 180°, was equal to the length of one segment. (Fig. 54.)

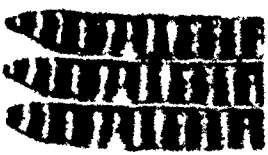

Fig. 54. Shift of Z-lines in a muscle fibril of *Dytiscus marginalis* on rotating by 0°, 45° and 90°.

These experiments show that the Z-stripes and herewith cross-striation are not conditioned by differences in the composition of the substance of the fibril but are expressions only of pe-

riodically changing optical properties of a system of spirals. The fibril-fragments, derived from different muscles, were partly right-, partly left-spirals.

Body muscle, with its rapid motion, must exert a big force within short periods of time. This makes close packing of the contractile substance necessary. This is not the case with the slowly working muscles of visceral organs. There is no need of such close packing and the spirals need not fit into each other, the spirals of one level will be in different phases and there will be no cross striation.[1]

One apparently serious objection may be raised against this theory of cross-striation: chemical analysis has shown that different substances (K, ATP, glycogen) are unequally distributed between Q and I bands. K and glycogen are located in the Q band while ATP is found in bigger concentration in the I band (CASPERSSON and THORELL). If their chemical composition is different, the bands must exist indeed. On considering this evidence it will be found that all the three substances have been detected by means of optical method, partly under the microscope (K, glycogen), partly by the spectrophotometer (ATP). But if these substances are present in the intermicellar splits or are present absorbed on the micels and are studied by optical methods, then they will show the same optical regularities as the micels themselves and their unequal distribution might be an optical illusion as well.

On closer consideration these chemical data rather support our theory. It has been found that all the three substances become evenly distributed between Q and J band on contraction, to return to their original place on relaxation. It is difficult to imagine a mechanism which would effect such a change. The change is readily explained by our model, because on contraction, owing to the bending of the actin-myosin system, the regularity of arrangement of intermicellar splits is disturbed. The fact, that ATP was found in the I band, K and glycogen in the Q band, may be due not only to the difference in adsorption to myosin, but also to the difference of the optical methods employed.

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[1] We can expect to find especially steep myosin-screws (high l/d relation of the double rod) and herewith wide Qu and I bands in the muscle of animal species which only swim at a uniform low speed (waterinsects), muscles of which are built to make rather extensive than intensive movements.

Conditions in resting muscle.

In order to understand muscular contraction, the transition from rest to activity, we have to know the conditions in the resting muscle: we have to know in what state myosin and actin are present and what is their relation. To approach this problem we must consider the basic data about muscle and try to correlate them with our observations.

The main morphological element of muscle is the contractile fibril which is suspended in the interfibrillary fluid. The physical properties of muscle depend in the first place on the state of these fibrils, and their changes, during contraction, must be due to changes in the fibril.

The fibrils contain about 33 % actomyosin. A 3 % F-actomyosin makes a fairly hard and elastic gel. A 33 % gel must be very hard. Resting muscle is soft. KÜHNE's observation may be recalled: he saw a nematode walking at ease through the fibrils of resting muscle. No nematode could walk at ease through a 33 % F-actomyosin gel.

Resting muscle contains thus either F-actin and myosin side by side (dissociated actomyosin), or else it contains G-actomyosin. It has been shown, however, that the G-actomyosin dissociates in presence of ATP at any salt concentration. ATP being present in muscle in rather high concentration. G-actomyosin has to dissociate. We are left thus with the sole possibility that resting muscle contains actin and myosin side by side and the question is whether the actin is present in its G- or F-form.

Actin. It has been shown that F-actin is broken up to G-actin during contraction and only F-actin is contractile. The G-actin has to be polymerised ere a new contraction can occur and the problem is whether this polymerisation occurs immediately after contraction — in which case the resting muscle contains F-actin — or at the beginning of the next contraction, in which case the resting muscle contains G-actin. The difference is rather important: should the resting muscle contain G-actin, then excitation would have to bring about its polymerisation, the polymerisation being followed by actomyosin-formation. If resting muscle contains F-actin, then, conditions evidently do not permit actomyosin-formation and the wave of excitation would have to bring about the change which conditions the union of actin and myosin.

It has been shown by STRAUB that the polymerisation of G-

actin depends, under certain conditions, on the balance of ions and it is believable that excitation brings about the ionic disturbance, necessary for the G—F-transformation.

The evidence, however, obtained till present, is in favour of the view that actin is polymerised immediately after contraction and resting muscle contains its actin in the F-form. At the actin-concentration of muscle, in presence of the ions of muscle, actin polymerises spontaneously at a high rate, especially in the presence of myosin. Moreover, at the end of contraction, the G-actin particles, originating from the breaking up of the F-actin, are in the right position for polymerisation. If actin were present in resting muscle in its G-form, then distilled water should extract G-actin from minced and uncontracted muscle and contraction would have to start with a rise of DR, corresponding to the F-actin formation. No such rise has been observed and distilled water does not extract G-actin. Resting muscle has also a considerable mechanic strength; G-actin could not contribute to this.

All evidence is thus in favour of the assumption that the actin, broken up during contraction, polymerises during relaxation and resting muscle contains F-actin. My further considerations will be based on this assumption, and on the following pages by "actin" F-actin and by "actomyosin" F-actomyosin will be meant.

Actomyosin. Muscle, under normal conditions, may exist in two different states: relaxed or contracted. In presence of ATP (normal muscle contains ATP) a system of actin and myosin may exist in two states: dissociated or contracted. Evidently, resting muscle contains its actomyosin in the first, contracted muscle in the last form.

Uncontracted and undissociated actomyosin is capable of existence only in absence of ATP. In muscle it can thus occur only under conditions where the ATP is exhausted. Such a condition is found *post mortem*, when muscle may be found in a third state: in *rigor*. Actomyosin, in presence of salts and in absence of ATP, is stiff and slightly contracted. Accordingly muscle in *rigor mortis* is stiff and slightly shortened.

Myosin. The dispersity of myosin depends, at a given pH, on the salt-concentration. One may ask to what dispersity the salt-concentration of muscle corresponds.

If muscle is heated rapidly till the proteins coagulate, a juice can be pressed out which contains most crystalloids of the muscle undiluted. The salt-concentration in this juice is higher than cor-

responds to the cellular fluid of the living cell, the adsorbed ions having been liberated.

If a myosin-thread is suspended in such a juice it does not swell or dissolve, the myosin remains highly associated. The same is true for a solution containing 0.105 *M* K, 0.006 *M* Ca and 0.012 *M* Mg as chlorides. According to DUBUISSON (17) this is the concentration of these cations in muscle, as calculated for 77 % of water.

In the muscle myosin must be present, thus, in a highly associated condition. Dissociation of actomyosin will not mean disassociation or disorganisation; the structures, formed by the association of myosin, will not be touched by the dissociation of actomyosin, which means no more than that certain links within the structure have ceased to be.

Metalmyosinate. At the high ATP-concentration of muscle (0.4 %, as calculated for 77 % of water), the zone of metal-saturation, in which myosin is capable of forming actomyosin must be very narrow. Outside this zone the actomyosin will be dissociated. One may ask, whether it is dissociated because the metal-saturation is above or below the contracting level, whether, in muscle, myosin is over- or under-saturated as regards contraction.

If the living frog's leg is perfused with RINGER's solution and the saline is suddenly replaced by distilled water, violent twitches occur which indicate that the myosin was oversaturated and the dilution has brought down the metal saturation to the contracting level. On the other hand ERDÖS showed that minced, fresh muscle does not contract in distilled water but it contracts in 0.02 *M* KCl, behaves thus as a salt-free actomyosin-thread in presence of ATP. But such a minced muscle is no more irritable and the loss of irritability shows that profound changes have taken place. It is possible that the loss of irritability was due to the decrease in metal-binding and the consequent loss of oversaturation.

Some, though inconclusive evidence may be obtained by testing the muscle-juice, prepared as described above. Actomyosin threads, if put into this juice, contract. This result, however, is only due to the slowness of diffusion. If the experiment is repeated in such a way that the thread is soaked with the juice at 0° (at 0° there is no contraction) and then brought to room temperature, it dissolves. If the juice is diluted with 30 % of

water it gives contraction only. The juice corresponds thus to dissociation but not far from the contracting level. The same is true for the salt-solution, mentioned above.

Muscular contraction.

Whether over- or under-saturated, the metal-saturation of myosin in resting muscle must be such as to exclude actomyosin-formation and excitation has to bring about the change which conditions it. It has been shown in this volume that very small changes in metal-saturation of myosin suffice to convert the dissociated system into a contracted one and *viceversa*. The loss or the gain of $^1/_2$—1 positive charge per myosin-unit suffice to induce this change. Since Ca and Mg are bound strongly by myosin and are thus immobilised, it will be the K which will condition contraction or relaxation by its motion.

It has been shown that the dissociation-curve of K-myosinate is rather flat below and steep above the isoelectric concentration. This means that relatively great changes of the K-concentration are needed to increase, and relative small changes are needed to decrease the metal-saturation of myosin to the contracting level. So, for instance, to make myosin take up $^1/_2$ K from KCl below the isoelectric concentration, we have to raise the KCl concentration from 0.006 to 0.025 *M*, have thus to concentrate the solution by 400 %. To make myosin give up one K above the isoelectric concentration, we have to lower the KCl concentration from 0.05 to 0.025 *M*, dilute thus our KCl solution by 100 % only.

This difference in the K-dissociation below and above the isoelectric saturation is still increased by the presence of bivalent ions. It has been shown that in muscle, in all probability, myosin is brought to the isoelectric metal-saturation by the bivalent ions, Ca and Mg. The K will be used only to charge the myosin positively, and this with a rather steep curve, which means that small changes in K concentration will suffice to bring about great changes in metal-saturation. According to the slope of this curve, dilution by 15 % will suffice to make one K come off. It has been shown in the experiment that, in presence of Mg and K, dilution by 10 % suffices to change complete dissociation of actomysoin into maximal contraction (Tab. VIII.)

On the other hand myosin, in presence of Ca and Mg, does not

bind any K below its isoelectric saturation. Thus, if muscle were metal-undersaturated, under normal conditions K could play no rôle in its contraction.

All this makes it highly probable that myosin, in resting muscle, does not unite with actin because it is over-charged with metal, and excitation has to bring about a loss of about 1 K per myosin unit to make it react with actin. In this stage the myosin is still positively charged and actomyosin formation is the union of positively and negatively charged particles.

If the K-actomyosinate dissociates K off, the ionisation of K has to induce electrostriction of water and herewith a volume-diminution of the system. If every 600 g of myosin gives off one K this has to cause a volume diminution of 0.025 cmm per g of muscle. It has been discovered by E. Ernst that muscular contraction is preceded by a volume-diminution, which, according to Ernst and Mórocz (106), in a short tetanus, is 0.02—0.1 cmm per g of muscle.

In what way the wave of excitation causes dissociation, we do not know. The simplest way to cause K-myosinate to give off metal is dilution, the simplest way to make it take metal up again is concentrating the solution.[1]

The very close packing in muscle creates special conditions. A hydrated protein binds about its equal weight of water. If the fibril contains 33 % of strongly hydrated actin-myosin and 67 % of water, then, at least half of this water has to be bound as hydrate water. This reduces the quantity of free water to 33 %. This means that intermicellar spaces must be very narrow. Since the spaces cannot be evenly distributed, at places there will be hardly any space at all. The strongly associated myosin micels, held together by cohesive links, cannot lay far apart anyway.

It has been shown by Banga that the metal dissociation curve of myosin is the same in the case of free and actinbound myosin,

[1] F. Guba has started experiments lately on the influence of poisons on the K-binding-capacity of myosin. He tested chloroform vapour, adrenaline (1 : 10,000), guanidine (1 : 1,000), coffeine (1 : 1,000), quinine (1 : 1,000), choline (1 : 1,000), and acetyl-choline (1 : 10,000 in presence of 1 : 10,000 physostigmine). They were inactive. The K-binding of myosin, in presence of ATP, however, was decreased specifically by acetylcholine in the positive region (above 0.025 M KCl). In this region the K-binding was increased by ATP and decreased to the original level by 1 : 100,000 acetylcholine. In presence of physostigmine this effect was permanent, while in absence of it the effect lasted only for a short time. Probably it was the binding of ATP which was inhibited by the acetylcholine. It will be remembered that at a certain K-saturation the decrease of the ATP-concentration also causes contraction (Tab. VIII). These effects demand a thorough study. Unfortunately external conditions did not permit us to complete these experiments.

in presence or absence of ATP. Actin-bound myosin, in presence of ATP, means contracted myosin. In contraction, thus myosin does not give up its metal, it gives up its hydrate-water only. This hydrate-water must cause a relatively great dilution of the intermicellar water and induce herewith contraction of neighbouring micels. This way contraction may become self-propagating. It is believable that the loss of K ions or ATP molecules, displaced by acetylcholine or torn off by an electric field, start up this process.

To bring this system to relaxation the intermicellar water will have to be concentrated. This process may be equally autocatalytic: a small local concentration may cause charging and hydration of the micel, which, in its turn, means further concentration. Thus, if these considerations are correct, the close packing must make the system highly metastable.[1]

According to this conception, the first change in muscular contraction would have to be a change in ionic distribution, a change which would have to declare itself in an electric potential and volume-contraction. The protein-structure, having lost metal ions, must be negatively charged relative to structures in rest. The next change would be actomyosin-formation which would have to cause a raise of pH. BANGA has made the observation that actomyosin-formation, in presence of KCl, is accompained by an increase in pH. If a slightly alcaline actin and myosin-solution are mixed, both containing some phenolphthaleine, but not enough OH ions to colour it, on the places of contract the solution turns red. Muscular contraction is preceded by an increase of pH, due to a hitherto unknown process (DUBUISSON 14).

The phosphatase-action of myosin.

BANGA has shown that twice recrystallised myosin has the same enzymic activity as in pure myosin. This seems to prove definitely that myosin is the enzyme itself. What renders difficult the acceptance of this conclusion is the low enzymic activity of myosin. If compared to other enzymes myosin is at least ten times more inactive; 10^5 g myosin split maximally 10 mols of ATP per second (139).

This low activity of myosin can easily be understood from the

[1] This metastability and self-propagation (irritability) must cease if the metal-saturation decreases or intermicellar splits open up and enter into equilibrium with the big volume of interfibrillar water.

point of view of physiology. Muscle, in monojodoacetic acid poisoning, is capable of about 70 full contractions. This means that the ATP present (0.3—0.35 %) can support as many contractions and that in one contraction, on average, one myosin micel of 10^6 splits one molecule of ATP. The low enzymic activity suffices for this action.

Difficulty arises if we consider the structure of myosin. The myosin micel is built of smaller globular units, probably of MW 70,000. If we consider myosin as consisting of particles of this size, then the "Wechselzahl" of these particles is at least one dimension too low if compared to other enzymes. Such an exception is highly improbable. It is more probable that only one out of the ten of these particles is enzymatically active, thus one out of the ten is not myosin at all, *in stricto sensu*, and the myosin micel is not a "substance" but a system, a higher organisation.

If our picture of the structure of the actin-myosin-spiral is correct, and the myosin-micels are attached to the actin thread by one end, then the narrow split between myosin and actin must be the seat of the most important changes. It is believable that the prosthetic enzymic group of the elongated myosin-micel is located at this end, adjoining actin. This is supported by the fact that resting muscle does not split ATP, its myosin being inhibited by Mg. The myosin becomes activated by the actomyosin-formation; it seems logical that its prosthetic group should lie where the myosin micel comes in touch with the actin.

The basic importance of ATP for contraction is beyond doubt. A high ATP-saturation of the myosin-micel is needed for contraction as well as for relaxation. The functional unit of the muscle is thus not actomyosin, but its ATP-complex. It is also known that there is a narrow relation between the quantity of the ATP split and the tension developed by the contracting muscle, and in end ATP has to pay the energybill of contraction. But whether the splitting of ATP is actually involved in the mechanism of contraction, we do not know. The evidence is rather against this assumption. It has been shown that contraction and relaxation are but precipitation and dissociation. Association, dissociation and precipitation are common experiences of colloidal chemistry and do not necessarily involve enzymic functions. It has also been shown that dissociation does not involve splitting of ATP and contraction is a related phenomenon.

The close parallel between contracting effect and hydrolyseability of different ADP:s only shows that the same pyrophosphate configuration conditions both qualities. It is quite believable that contraction (actomyosin-formation) in muscle is induced by the dilution, relaxation (actomyosin-dissociation) by the concentration of the intermicellar fluid, by means of the consequent release or binding of K. Actomyosin-formation releases the myosin from its Mg-inhibition, and the contracting muscle begins to split ATP. It seems not impossible that it is the ATP-splitting, the heat of which removes the water and concentrates the intermicellar fluid by means of the thermoosmotic effect of J. Ernst and Koczkas.[1]

The consequent dissociation of actomyosin brings the ATP-splitting to a standstill again. If this conception is correct, muscle works as a heat-engine.[2]

Myosin and actomyosin still present a great number of most fascinating problems and "the problem of muscular contraction" is still unsolved. What emerges clearly from the mass of observations is that the functional unit of the muscle is not actomyosin but its ATP-complex, the unit of protein and nucleotide. There seems also no doubt that ions play a basic rôle in this function and determine the two basic conditions of living matter: rest and activity. Ions were the only powerful tools which life found in the ocean where it originated and it seems to be working with these tools up to the present day.

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[1] Z. f. Physik, *109*, 625, 1938.
[2] The energy of the splitting of ATP suffices to do the osmotic work required.

PART VIII.

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AUS DEM MEDIZINISCH-CHEMISCHEN INSTITUT DER UNIVERSITÄT
IN HELSINGFORS (PROF. P. E. SIMOLA)

TIEREXPERIMENTELLE UNTERSUCHUNGEN ÜBER DEN CALCIUM- UND PHOSPHORSTOFFWECHSEL IN KNOCHEN MIT BESONDERER BERÜCKSICHTIGUNG DER WIRKUNG DES D-VITAMINS

VON

BERNHARD LANDTMAN

ACTA PHYSIOLOGICA SCANDINAVICA
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INHALTSVERZEICHNIS.

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VORWORT.

Die Anregung zu der vorliegenden Arbeit ist mir von Herrn Professor Dr. med. et phil. P. E. SIMOLA, dem Vorstand des Medizinisch-chemischen Instituts der Universität in Helsingfors, gegeben worden. Im Vorsommer des Jahres 1941 war bereits ein grosser Teil der Versuche ausgeführt, aber die Arbeit musste infolge des Kriegsausbruches abgebrochen werden. Die Vollführung derselben ist auf Grund der gegenwärtig herrschenden Verhältnisse bedeutend verzögert worden. Die experimentellen Untersuchungen fanden im Herbst 1943 ihren Abschluss. Bis zu demselben Zeitpunkt erstreckt sich auch die so gut wie gesamte in der Arbeit behandelte Fachliteratur. Der grösste Teil der Abhandlung ist während meines Dienstes als Bataillonsarzt im jetzigen Kriege geschrieben worden.

Gelegentlich der Abschliessung dieser Arbeit ist es mir eine angenehme Pflicht meinen tiefgefühlten Dank an meinen verehrten Lehrer, Herrn Professor P. E. SIMOLA für die gütige Überlassung dieses Themas, sowie für die grosse Bereitwilligkeit und die inspirierenden Ratschläge, mit denen er mir bei der Gestaltung der Arbeit während der ganzen Zeit beigestanden hat, in erster Reihe auszusprechen. Auch für die Gelegenheit meine Untersuchungen in dem von ihm geleiteten Laboratorium ausführen zu dürfen, sowie für das zu meiner Verfügung freigebig gestellte Tiermaterial und die Apparaturen befinde ich mich ihm gegenüber in grosser Dankes-Schuld.

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Ferner bin ich dem Vorstand des Sero-bakteriologischen Instituts der Universität in Helsingfors, Herrn Professor Dr. K. O. Renkonen, sowie Herrn Dr. U. P. Kokko für ihre Ratschläge betreffend die in dieser Arbeit ausgeführten bakteriologischen Versuche ergebensten Dank schuldig.

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Syväri, im April 1944.

Bernhard Landtman.

I. Einleitung.

Der Verknöcherungsvorgang, sowie die im Zusammenhange mit demselben stehenden Erscheinungen im Knochengewebe, stellen seit lange her den Gegenstand zahlreicher Untersuchungen dar. Mit dem Fortschritte der Vitaminforschung — insbesondere nach der Entdeckung des Vitamin D — ist diesem Problem ein stetig wachsendes Interesse zum Teil gekommen. Obgleich die Literatur auf diesem Gebiete sehr umfangreich ist, sind bisher nur wenige Untersuchungen ausgeführt worden, die dem Zwecke dienten den Calcium- und Phosphormetabolismus in normalem Knochengewebe, sowie den Zusammenhang zwischen diesem Stoffwechsel und den verschiedenen die Ossifikation beeinflussenden Faktoren vermittelst in vitro und auf rein chemischem Wege ausgeführter Versuche zu beleuchten. Es erschien demnach von Intresse dem genannten Problem ein näheres Studium von diesem Gesichtspunkte aus zu widmen.

Die vorliegende Arbeit wurde im Jahre 1940 begonnen und stellt eine Fortsetzung der in den Jahren 1936—1940 von v. Kraemer und Landtman unter der Leitung von Simola ausgeführten Untersuchungsserie dar.

Die Art dieser Arbeit lässt eine Einteilung der Untersuchungen in zwei Abteilungen als berechtigt erscheinen. In der ersten Abteilung ist die Aufmerksamkeit dem Studium des Calcium- und Phosphorstoffwechsels in normalem Knochengewebe gewidmet. Um einen so vollständigen Einblick wie möglich in diese Frage zu gewinnen, wurde sowohl die Aufnahme, wie auch die Retention von Calcium und Phosphor im Knochengewebe beobachtet. Diese letztere Seite des Calcium- und Phosphormetabolismus ist, wie wichtig und interessant sie auch erscheinen mag, bisher noch nicht näher untersucht worden.

Ausgehend von den Versuchen mit normalem Knochengewebe wurde in dem nachfolgenden Teil der Arbeit die Wirkung von Vitamin D auf den genannten Stoffwechsel im Knochengewebe untersucht. Hierbei wurde das Problem von zwei Seiten beleuchtet. Zuerst wurde die Aufnahme und die Retention von Calcium und Phosphor im Knochengewebe nach Verabreichung grosser Mengen D-Vitamin an die Versuchstiere untersucht. Von dergleichen, bei D-Hypervitaminose ausgeführten Untersuchungen hat die Literatur bisher nichts zu berichten.

Des weiteren wurde der entsprechende Calcium- und Phosphor-Stoffwechsel bei D-Avitaminose studiert. Die bei diesen Untersuchungen angewandten Tiere waren mit der rachitogenen Kost Mc Collums aufgezogen. Diese ergänzenden Versuche hatten als ihren Zweck soweit wie möglich festzustellen, ob die rachitischen Veränderungen im Skelett zum Teil auf irgendeine Minderwertigkeit in der Aufnahme und Retention von Calcium im Knochen-Gewebe im Vergleich mit normalen Verhältnissen zurückgeführt werden könnten.

Ursprünglich hatte ich die Absicht bei diesen Untersuchungen mich der Einheit wegen nur von Ratten als Tiermaterial zu bedienen. Da es sich jedoch unter den gegenwärtig herrschenden Umständen als schwer erwies eine genügende Menge dieser Tiere aufzutreiben, musste das Tiermaterial mit Meerschweinchen und Kaninchen vervollständigt werden. Einige der Versuchsserien sind deshalb verhältnismässig kurz ausgefallen, jedoch könnten andererseits die jeweiligen Ergebnisse infolge eines solchen Verfahrens vielleicht eine mehr weitreichende Geltung haben.

Ehe ich an einen Bericht über die Arbeitsmethode und die Ergebnisse der Versuche eingehe, erscheint es angebracht eine Zusammenfassung der wichtigsten Gesichtspunkte, von denen aus das Ossifikationsproblem, sowie der Wirkungsmechanismus des D-Vitamins in diesem System beurteilt werden, zu machen. Gleichzeitig wird auch über die wichtigsten in vitro ausgeführten Versuche auf diesem Gebiete näherer Bericht erstattet.

II. Literaturübersicht.

A. Die Chemie der Ossifikation.

Die Histogenese der Knochenbildung ist genau untersucht und im grossen ganzen bekannt. Die bei der Knochenbildung vor sich gehenden biochemischen Prozesse dagegen, obgleich sie den Gegenstand mannigfacher Forschungen gebildet haben, haben sich noch immer zum grossen Teil unserem Wissen entzogen. Auf verschiedene, die Knochenbildung betreffende Fragen haben wir noch keine endgültige Antwort gefunden, sei es weil unser Kenntnis von den Eigenschaften der Knochensalze nicht vollständig ist, sei es infolge unserer ungenügenden Bekanntschaft mit dem Mechanismus der Aufspeicherung dieser Salze im Knochen. Die Wichtigkeit, das Knochen-Gewebe als ein lebendes Organ, und nicht als eine nach seiner Bildung mehr oder minder unveränderliche Masse zu betrachten, ist in der letzten Zeit immer stärker hervorgehoben worden. Seine Bedeutung als Mineralspeicher für den Körper neben seiner Funktion des Stützgewebes — der augenscheinlich wichtigsten Aufgabe der Knochen — darf keineswegs unterschätzt werden. Da etwa 99 % des Calciums und 65 % des Phosphorvorrates des Körpers in den Knochen aufgelagert sind, versteht man im Kenntnis der diesen Stoffen bei den verschiedenen Funktionen des Körpers zukommenden Rolle, dass diese Eigenschaft der Knochen von weitläufiger Bedeutung ist. Die Knochensubstanz gehört zu den lebenden Organen und befindet sich als ein solches in einem stätigen Austausche mit dem Blute. Policard und Roche (1937) Kennzeichnen diesen Umstand wie folgt: »La substance osseuse est un des materiaux les plus labiles de l'organisme».

Die Knochensälze sind nicht nur auf die organische Matrix aufgelagert, sondern auch auf dieselbe fixiert. Unter normalen Verhältnissen wird der Stoffwechsel in dem Knochengewebe durch ein harmonisches Zusammenspiel zwischen der Aufnahme, Fixierung und Abgabe der dasselbe bildenden Bestandsteile charakterisiert. Sollte jedoch dieses Gleichgewicht, wie, z. B., bei kachektischen Zuständen, Störungen erfahren, treten in dem Knochengewebe, gleich den übrigen Organen, dystrophische Veränderungen auf, welche sich durch eine Demineralisierung äussern. Auch unter normalen Umständen findet in den Knochen sowohl ein Aufbau, wie ein Niederbrechen statt. Die Demineralisierung der Knochensubstanz

wird auf zwei Weisen, nämlich durch Osteoklasie und Osteolyse physiologisch ermöglicht, von denen die erstere ein Ergebnis der phagocytären Tätigkeit der Osteoklasten ist. Bei der Osteolyse lösen die Beinsalze sich auf und wandern in die umgebenden Gewebe aus (Halisteresis). Das Fixieren von Knochensalzen an die Matrix, sowie das Abgeben derselben stellt ein bisher in vielen Punkten noch ungeklärtes Problem dar.

Wenn man von Knochengeweben spricht, ist man also so gut wie berechtigt von einem veränderlichen Organ zu reden. In diesem Lichte sind auch die Schwierigkeiten der Beobachtung und der Beurteilung der in der Ossifikation einbegriffenen Prozesse, sowie der verschiedenen Versuchs-Ergebnisse erklärlich.

Bei der Beurteilung des anorganischen Stoffwechsels im Knochen-Gewebe stellen sich die folgenden hauptsächlichen Fragen auf:

1) Von welcher Natur sind die Knochensalze?

2) In welchen Vereinigungen kommen die wichtigsten anorganischen Komponente im Blute vor?

3) Wie ist der Aufnahmemechanismus beschaffen und welche Faktoren dürften denselben beeinflussen?

1. *Die Chemie des fertigen Knochengewebes.*

Das eigentliche Knochengewebe, abgesehen vom Mark, den Nerven und den Blutgefässen, besteht aus einer Grundsubstanz und aus in dieselbe eingebetteten Knochenzellen. Die Grundsubstanz enthält zwei Hauptbestandteile, nämlich die organische Knochenmatrix, sowie die Knochen-Salze, welche spätere die sogenannte Knochenerde darstellen.

Der normale Knochen, einschliesslich Mark, enthält rund 50 % Wasser, 16 % Fett, 12 % organische Grundsubstanz, und 22 % Knochenerde. Der Wasser- und Fettgehalt schwankt in grossem Massen in den verschiedenen Knochen eines und desselben Individuums, und hängt ausserdem auch vom Alter und dem Geschlecht ab (Hammett 1925). Markfreie Knochen enthalten etwa 20—25 % Wasser.

Die Zusammensetzung der Trockensubstanz ist dagegen verhältnissmässig konstant. Sie besteht zu 30—40 % aus organischen Stoffen, von welchen 15—25 % Fett sind, und zu 60—70 % aus anorganischem Material.

Organische Substanz. Die organische Knochenmatrix besteht hauptsächlich aus Ossein, welches mit Bindegewebskollagen sehr nahe verwandt ist, und ausserdem aus kleineren Mengen Osseomukoid und Osseoalbumoid. In der letzten Zeit hat unter den im Knochengewebe vorkommenden organischen Bestandsteilen die Citronensäure ein steigendes Interesse erweckt. Dickens (1941) hat z. B. gezeigt, dass die harte Knochensubstanz etwa 70 % der im Organismus befindlichen gesamten Citronensäuremenge enthält. Der Knochenmark und der Knorpel haben dagegen nur einen verhältnismässig geringen Citronensäuregehalt aufzuweisen. Nicholaysen und

NORDBØ (1943) haben bei der Analyse frischer Knochensubstanz von Ratten eine Citronensäuremenge von bis 0.66 % gefunden.

Anorganische Substanz. Die Knochensalze legen sich auf der kalkaffinen Knochenmatrix ab. Auf welche Weise diese Vereinigung vor sich geht ist nicht näher bekannt, aber man hat öfters die Auffassung ausgesprochen, dass es sich hier um eine Adsorptionsbindung handelt. Da die anorganische Substanz für das Knochengewebe charakteristisch ist und funktionell den wichtigsten Teil desselben darstellt, ist sie seit der grundlegenden Untersuchungen von BERZELIUS und HOPPE-SEYLER der Gegenstand der meisten und eingehendsten Studien gewesen.

GABRIEL (1894) gibt die folgenden Werte bei der Analyse der Knochen-Asche verschiedener Tiere in % auf:

	Der Mensch	Das Rindvieh	Die Gans
Ca O	51.31	51.28	51.01
Mg O	0.77	1.05	1.27
K_2O	0.32	0.18	0.19
Na_2O	1.04	1.09	1.11
Kristallwasser	2.46	2.33	3.05
P_2O_5	36.65	37.46	38.19
CO_2	5.86	5.06	4.11
Cl	0.01	0.04	0.06
Konstitutionswasser	1.32	1.37	1.07
	99.74	99.86	100.06

Andere Minerale, wie z. B. Fe, F und Pb, sind in nur unbedeutenden Mengen vorhanden.

Auffallend sind die beinahe konstanten Werte der wichtigsten anorganischen Komponenten Calcium und Phosphor in den Knochensubstanzen der verschiedenen Tierarten, welche Konstanz u. a. auch von MORGULIS und JANEZEK (1931) bestätigt worden ist.

Während im fertig ausgebildeten Knochen die Calcium- und Phosphor-Konzentration keinen nennenswerten Schwankungen unterworfen ist, ist dieses für die Kohlensäure in der Knochensubstanz nicht zutreffend. KRAMER und SHEAR (1929), wie auch LOGAN (1935) haben beispielsweise bei der Untersuchung von »fresh bone» gefunden, dass der Carbonatgehalt in den Knochen mit dem Alter zunimmt. Weder hat der Kohlensäure-Gehalt in den Knochen verschiedener Tierarten dieselben ziemlich unveränderlichen Werte wie Calcium und Phosphor aufzuweisen, sondern er scheint in einem grossen Ausmasse variieren zu können.

2. *Veränderungen in der Mineralienzusammensetzung der Knochensubstanz.*

Wir wissen, dass die anorganische Zusammensetzung des Knochen-Gewebes nach Beendigung der primären Ossifikation nicht unverändert ver-

bleibt. Diese »Mutabilität» ist für das Knochengewebe kennzeichnend. Oben ist schon die Beobachtung, dass der Carbonatgehalt der Knochen-Substanz sich mit dem Alter erhöht, angeführt worden. Es ist jedoch nicht bekannt, ob dieses eine Folge der unmittelbaren Aufnahme von $CaCO_3$ ist, oder ob die Zunahme auf Veränderungen in der anorganischen Mineral-Struktur des Knochengewebes zurückzuführen ist. Die ersterwähnte Möglichkeit wird jedoch von POLICARD und ROCHE (1937) als die wahrscheinlichste erachtet. Die Aufnahme von solchen Stoffen wie Pb, Ag, Fe, Ra u. s. w. ist von dem Gebiete der Berufskrankheiten her bekannt, vom Mechanismus dieser Fixierung hat man jedoch von biochemischem Gesichtspunkte aus kein näheres Kenntnis. Im Zusammenhange mit der Fossilisation stehende Veränderungen der Knochenmineralien sind u.a. von de JONG (1926) im Röntgenspektrogramm beobachtet worden, wobei er das Vorkommen verschiedener Stoffe, wie z. B. F, in recht grossen Mengen in der Knochensubstanz fossiler Tiere nachweisen konnte. In diesem Zusammenhange dürfte man die Beobachtung erwähnen, dass Phosphor in den Knochen *teilweise von seiner radioaktiven Isotope substituiert werden* kann. Diese vor etwa 20 Jahren zuerst von HEVESY beobachtete Erscheinung ist später von COHN und GREENBERG (1938, 1939) ausführlich beschrieben worden. Bei der Verabreichung von radioaktivem Phosphor per os an Ratten wurde bei elektroskopischen Untersuchungen beobachtet, dass dieser Phosphor sich im Organismus ebenso wie seine normale Isotope verteilte, und zwar nahm bei diesen Experimenten von allen Organen das Knochengewebe die grösste Menge radioaktiven Phosphors auf, welcher nachträglich eliminiert und wieder von gewöhnlichem Phosphor ersetzt wurde. Diese Beobachtung ist vom osteogenetischen Gesichtspunkte aus insofern interessant, dass sie zeigt, dass die Komponenten der Knochen-Mineralien gegen andere so zu sagen ausgetauscht werden können.

3. *Calcium- und Phosphorverbindungen im Knochengewebe.*

Seit der Erfahrung, dass Calcium- und Phosphor den Hauptbestandteil der Mineralien in Knochen ausmachen, hat die Forschung sich mit der Frage beschäftigt, in welcher, oder in welchen Vereinigungen diese Elemente im Knochengewebe vorkommen. Besteht also überhaupt eine ein spezifisches »Knochensalz» darstellende Verbindung von Calcium und Phosphor? Einige Beobachtungen deuten darauf hin, dass dieses möglich ist. Es ist nämlich gezeigt worden, dass Calcium und Phosphor in den Knochen in einem beinahe konstanten, vom Entwicklungsstadium und dem Mineraliengehalt in der Diät unabhängigen Verhältnis vorkommen. (HOLMES, PIGOTT und CAMPBELL 1931). Auch bei Rachitis (HOWLAND, MARIOTT und KRAMER 1926) und bei D-Hypervitaminose (KRAMER, SHEAR und MCKENZIE 1929) verbleibt dieses Verhältnis ziemlich unverändert. In den Ergebnissen der obenerwähnten Forscher schwankt der Wert des Verhältnisses vom rezidualen Calcium und Phosphor zwischen 1.93 und

2.09. Diese relative Konstanz des Calcium- und Phosphorgehaltes im Knochengewebe hat zu einer Anzahl von Vermutungen über die Verbindungen, welche diese Elemente miteinander in den Knochen bilden, Anlass gegeben.

Sekundäres Calciumphosphat ($CaHPO_4$). Die Theorie, dass $CaHPO_4$ eine Zwischenverbindung bei der Bildung von einem mehr basischen Calcium-Phosphatsalze ist, wurde von SHEAR und KRAMER (1928) entwickelt, welche ihre Annahme auf die Beobachtung stützten, dass bei der Mischung von Ca und PO_4 enthaltenden Lösungen die initiale Fällung öfters $CaHPO_4$ ist. Dieses Salz soll primär in den Knochen aufgenommen werden und sich also stets in denselben befinden, um nachträglich in eine solidere Phase, deren Zusammensetzung sich $Ca_3(PO_4)_2$ nähert, zu übergehen, vielleicht durch die Wirkung eines Mechanismus, der in vitro beim Schütteln von $CaHPO_4$ in Carbonate oder Bicarbonate enthaltenden Lösungen die Fällung von $Ca_3(PO_4)_2$ veranlasst (KLEMENT 1928). SHEAR und KRAMER stützen des weiteren ihre Theorie auf die Beobachtung, dass die Calcification, sei es in vivo oder in vitro, nur in den Fällen nachgewiesen werden konnte, wo das empirische Ionprodukt $(Ca^{++}) \times (HPO_4^{=})$ im Blute oder in der Inkubationsflüssigkeit bemerkbar grösser ist als das Löslichkeits-Produkt von $CaHPO_4$.

Tertiäres Calciumphosphat [$Ca_3(PO_4)_2$]. Der Annahme, dass das hauptsächlichste Knochensalz aus einer Mischung von $Ca_3(PO_4)_2$ und $CaCO_3$ besteht, sind in der letzten Zeit HOLT (1925), sowie MAREK, WELLMAN und URBÁNYI (1935) beigetreten. Nach HOLT dürften 85 % der anorganischen Knochenfraktion aus $Ca_3(PO_4)_2$ und 14 % aus $CaCO_3$ bestehen. Nehmen wir an, dass im grossen ganzen alle Kohlensäure mit Calcium in der Form von $CaCO_3$ gebunden ist, und bringen wir dieses durch Kohlensäure gebundenes Calcium vom gesamten Calcium in Abzug, so ist das Verhältnis zwischen diesem sogenannten rezidualen Calcium und Phosphor in dem Knochengewebe, wie bereits erwähnt, verhältnismässig konstant. $Ca_3(PO_4)_2$, wo diese Zahl 1.94 ist, entspricht diesem Verhältnis, was nach der Meinung von HOWLAND, MARIOTT und KRAMER (1926) überaus zu Gunsten der Anwesenheit einer solchen Calcium-Phosphor-Verbindung im Knochengewebe spricht. Von $CaHPO_4$ als von einem schlussgültigen Knochensalz könnte dagegen nicht die Rede sein, da in demselben das Verhältnis zwischen Calcium und Phosphor nur 1.29 ist.

Es muss jedoch hervorgehoben werden, dass $Ca_3(PO_4)_2$ aus Knochen nicht isoliert werden konnte, und dass dieses Salz als Mineral nicht bekannt ist (TAYLOR und SHEARD 1929). Nach DANIEL und FRÖLICH (1930) ist $Ca_3(PO_4)_2$ eine Verbindung, die, in Wasser gelöst, in Apatit übergeht.

Apatit. Der nunmehr allgemein angenommenen Auffassung nach hat das wichtigste endgültig ausgebildete Knochensalz die Struktur von Apatit. Man ist jedoch verschiedener Ansicht darüber, ob man hier mit einem Carbonat- oder Hydroxylapatit zu tun hat. Diese zwei Auffassungen werden von einer dritten verglichen, nach welcher das wichtigste Knochensalz aus einem multiplen Apatit bestehen soll.

Carbonatapatit [$Ca_3(PO_4)_2 \cdot 3\,CaCO_3$]. Diese Formel für Knochensalz wurde zuerst von WERNER (1907), dessen Ansicht nach diese Verbindung das Mineral Podolit vorstelle, vorgeschlagen, und diese Annahme wurde von GASSMANN (1910) unterstützt. Seine Theorie gründet GASSMANN auf die Beobachtung, dass beim Rachitis Calcium, Phosphor und Kohlensäure in den Knochen im Verhältnis 10 Ca: 6 PO_4 : 1 CO_3 abnahmen, was für das Dasein dieses Komplexes sprechen dürfte, denn nur eine so geformte Verbindung könnte eine Reduktion dieser Stoffe in dem angeführten Verhältnis ermöglichen.

Die wichtigsten Argumente zu Gunsten der Apatit-Theorie sind in der letzten Zeit von röntgenspektrographischen Untersuchungen gegeben worden. So haben u. a. ROSENBERRY, HASTINGS und MORSE (1931) gefunden, dass Knochen im Röntgenspektrogramm eine kristallinische Struktur aufweisen, die unverkennbar dieselbe wie die dem Apatit eigene ist. Emaille weist ebenfalls dieselbe Struktur auf. Gleichzeitig versuchten sie auch in Beweis zu bringen, dass $CaHPO_4$ und $Ca_3(PO_4)_2$ in dem reifen Knochen im freien Zustande nicht vorkömmen. Sie gaben für das Knochensalz die Formel $n \cdot CaCO_3 \cdot nCa_3(PO_4)_2$, wo n gleich 2—3 ist, welche Struktur-Formel u. a. auch in LOGAN (1935) Stütze gefunden hat.

Hydroxylapatit [$Ca_3(PO_4)_2 \cdot Ca(OH)_2$]. Bei der Analyse von Knochen-Asche hat GABRIEL (1894) einen Überschuss (6 %) von Base den Säureequivalenten gegenüber festgestellt und diese Beobachtung wurde auch von KLEMENT (1928) sowie von MORGULIS und JANECEK (1931) bestätigt. LOGAN (1935) hat »fresh bone» auf anorganische Base, sowie auf Phosphat und Carbonat untersucht und ebenfalls einen Überschuss an Base gefunden. KLEMENT, der in der letzten Zeit die Hydroxylapatit-Struktur verfochten hat, vermutet, dass der grösste Teil von Na, K und Mg in den Knochen an CO_2 gebunden ist. Sollte dieses der Fall sein, dürfte der Ansicht von KLEMENT nach ein allzu kleiner Teil der Kohlensäure übrig bleiben, um das Radikal $CaCO_3$ der Carbonatapatit-Formel bilden zu können. Auf Grund von röntgenspektrographischen Untersuchungen kommen TRÖMEL und MÖLLER (1929) zur Schlussfolgerung, dass die Knochensubstanz das Diagramm von Hydroxylapatit ergiebt.

Multipler Apatit [$n.\,Ca_3\,(PO_4)_2 \cdot Ca\,x_2$]. Eine der Schwierigkeiten, auf die man bei der genauen Röntgenanalyse der Knochen stösst, hat ihren Grund in den kleinen Dimensionen, etwa 10^{-5}—10^{-6} cm, der Kristallelemente, welche die Reflexionslinien unscharf machen (KLEMENT und TRÖMEL 1932). DE JONG (1926), sowie TAYLOR und SHEARD (1929) haben demnach vorgeschlagen, dass die Knochensalzstruktur am besten durch die allgemeine Formel $3\,Ca_3\,(PO_4)_2 \cdot Ca\,X_2$ ausgedrückt werden dürfte, worin X gewöhnlich CO_3, F_2, $(OH)_2$, O oder SO_4 ist, und in der an Stelle von Ca eine kleine Menge Mg eingesetzt werden kann. Die typischen Mineralien von diesem Typus sind Dahlite und Fluorapatit.

HUGGINS (1937) ist der Annahme, dass das hauptsächlichste Knochen-Salz eine Mischung von Carbonat- und Hydroxylapatit enthalten dürfte. Gemäss POLICARD (1937) soll das Salz aus fixiertem Apatit und darüber hinaus aus einer veränderlichen Menge $Ca_3\,(PO_4)_2$ bestehen.

4. *Calcium und Phosphor im Blute.*

Die Ossifikation wird dadurch ermöglicht, dass vom Blute und der Gewebelympha »Bausteine» der Proteinstroma zugeführt werden. Von den Haupbestandteilen der Knochensälze werden Calcium und Phosphor in das Blut vorwiegend im alimentaren Wege durch den Darm aufgenommen. Kohlensäure dagegen entsteht vorzugsweise als ein Produkt der Oxydation und ist also stets im Organismus vorhanden.

Calcium im Blute. Der normale Calciumgehalt im Blutserum des Menschen ist, wie bekannt, etwa 10—11 mg %. RONA und TAKAHASHI (1911, 1913) haben als die ersten experimentelle Sicherheit dafür erbracht, dass ein Teil des Serumkalkes eine für Protein undurchdringliche Dialyse-Membrane nicht ohne weiteres passieren kann, wogegen der Rest dieselbe durchdringt. Ihre Annahme, dass das nicht diffusible Calcium an Protein gebunden ist, hat später allgemeine Bestätigung gewonnen. Diese Fraktion bildet annähernd die Hälfte des Serumkalkes (RONA und TAKAHASHI (1913) SCHMIDT und GREENBERG 1935, LEBEL 1939 u. a.). Die Ansichten betreffend die Zusammensetzung der diffusiblen Calcium-Fraktion sind jedoch noch immer geteilt, es wird jedoch allgemein angesehen, dass ein Teil dieser Fraktion aus ionisiertem Calcium, C^{++}, besteht. Bei der Bestimmung der Konzentration von Calcium in dem Dialysat ist man jedoch auf Schwierigkeiten gestossen. Ältere, auf elektrochemische Beobachtungen gegründete Untersuchungen ergaben, was biologische Lösungen betrifft, unsichere Resultate.

McLEAN und HASTINGS (1934—35) sind in der letzten Zeit auf eine befriedigende Methode für die Bestimmung von Ca^{++} im Serum gekommen. Untersuchungen ergaben, dass für die Ionisierung des Calciums das Gleichgewicht zwischen Calcium und Protein primär bestimmend ist. Dieser Gleichgewichtszustand kann laut dem Grundsatze des Massenwirkungs-Gesetzes ausgedrückt werden, wobei die Proteinmolekülen bei ihrer Vereinigung mit Calcium als divalente Anionen wirken würden.

Die empirische Formel $\frac{(Ca^{++}) \times (Prot^{=})}{(Ca\ Prot)} = K = 10^{-2,22}$ hat mc LEAN und HASTINGS die Möglichkeit gegeben Ca^{++} im Blutserum mit einer Sicherheit zu bestimmen, welche ebenso zuverlässig ist wie die direkte Messung mit ihrer vorherigen »frog heart» Methode. Mc LEAN und HASTINGS haben gefunden, dass im normalem Serum die Konzentration des ionisierten Calciums 4.4—5.6 mg % ist, welche Beobachtungen neulich u. a. von HERLITZ (1942) bestätigt worden sind. Durch Einsetzen der analytischen Werte des totalen Calciums und des totalen Proteins in die obenangeführte Formel, hat er einen Mittelwert von 5.25 mg % für Ca^{++} im Serum normaler erwachsener Menschen erhalten. Gleichzeitig hat er beobachtet, dass die Konzentration von Ca^{++} im Serum mit dem Alter steigt, weil Neugeborenen die niedrigsten Werte aufwiesen.

LEBEL (1939) hat auf Grund seiner Beobachtungen über die Löslichkeit

des Calciumjodats den Gehalt von Ca^{++} im Serum von Menschen als 5.4—6.1 mg % bestimmt.

Ein Teil der schon früher ausgeführten Untersuchungen spricht jedoch dafür, dass die diffusible Calciumfraktion des Serums noch eine weitere Komponente aufzuweisen hat, die von einem Calciumkomplex vertreten werden dürfte. KLINKE (1928) hat somit gefunden, dass der Calciumgehalt im Serum beim Schütteln mit verschiedenen positiven Adsorbentien, wie z. B. $Ca_3(PO_4)_2$ und $BaSO_4$, abnimmt, welche Beobachtung für das Vorhandensein einer negativ geladenen Calciumverbindung sprechen dürfte. SHEAR, KRAMER und RESNIKOFF (1929), sowie v. BEZNÁK (1930) u. a. sind der Ansicht, dass in diesem Komplex das Calcium mit Citrat gebunden ist. BENJAMIN und HESS (1933), welche die Methodik von KLINKE entwickelt haben, behaupten vier Calciumfraktionen im Serum, zwei diffusible und zwei nicht diffusible, vermittelst Adsorption und Ultrafiltrierung ausscheiden zu können. Zwei Drittel des diffusiblen Calciums sollen aus einem adsorbierbaren Calcium-Phosphor-Komplex und der Rest aus ionisiertem Calcium bestehen. Drei Viertel des Calciums der nicht diffusiblen Calciumfraktion sollen an Protein gebunden sein, wogegen ein Viertel sich wohl adsorbieren lässt, aber nicht ultrafiltrierbar ist. BENJAMIN und HESS sagen, dass sie das Vorkommen des Calcium-Phosphor-Komplexes auch in anorganischen Lösungen haben nachweisen können, und schreiben demselben eine grosse Bedeutung für die Ossifikation zu. Späterhin haben DUCKWORTH und GODDEN (1936) ähnliche Untersuchungen mit der Barium-Sulphat-Methode ausgeführt und sind zu Ergebnissen gekommen, welche diejenigen von BENJAMIN und HESS unterstützen.

Das Vorkommen einer Calciumkomplexverbindung im Serumdialysat ist jedoch von verschiedenen Forschern, wie LASKOWSKI (1933), SCHMIDT und GREENBERG (1935) in Zweifel gezogen worden. Die letzteren haben gefunden, dass positive Calciumionen unter gewissen Bedingungen von positiven Adsorbentien, wie z. B. $BaSO_4$, infolge von Veränderungen in deren Ladung adsorbiert werden können. Sie haben nämlich wahrgenommen, dass die Ladung von $BaSO_4$, in der Anwesenheit von Phosphat- oder Sulphat-Ionen sich in negativer Richtung verändert. Diesen Forschern nach soll das Blut genügend an Phosphat enthalten, um eine Veränderung der Ladung von $BaSO_4$ in negativer Richtung zu bewirken.

Phosphor im Blute. Wie bekannt, beträgt der normale Gehalt von anorganischem Phosphor in der Blutplasma etwa 3—5 mg %. Neulich haben GÖBEL und JANDA (1942) Untersuchungen vorgenommen, welche darauf hindeuten, dass der ehemalig als eine anorganische Fraktion angesehene Phosphor bis auf 70 % aus Kreatinphosphorsäure bestehe, und dass nur 1.0—1.8 mg % des im Blute befindlichen Phosphors »wahrer anorganischer Phosphor» sei. Diese Beobachtungen, welche für Vollblut gültig sind, dürften noch auf ihre Bestätigung durch Nachprüfungen warten.

Die gesamte anorganische Phosphorfraktion des Blutplasma ist tatsächlich ultrafiltrierbar und diffusibel, wenn das Blut Calcium und Phosphat innerhalb normaler Grenzen enthält (LOGAN 1940). Nach BENJAMIN

und HESS (1933) soll der anorganische Phosphor im Plasma in zwei Formen vorkommen, und zwar entweder in Verbindung mit Calcium, oder als ionisiertes Phosphat. In der Tat scheinen jedoch Calcium und Phosphor eine kleine Menge nicht dissoziierten Salzes bei einer niedrigeren Konzentration, als die, bei welcher Fällung stattfindet, zu bilden, jedoch ist diese Menge so klein, dass sie sich kaum mit den zu Gebot stehenden Methoden berechnen lässt (LOGAN 1940). Sollte dagegen die Konzentration von Calcium oder Phosphor in der Blutplasma sich abnorm erhöhen, wird kolloidales Calciumphosphat gebildet (LASKOWSKI 1933, MC LEAN und HINRICHS 1938).

5. *Über den Mechanismus der Aufnahme von Calcium und Phosphor in Knochengewebe.*

Obwohl der Mechanismus der Knochenbildung der Gegenstand eingehenden Studiums gewesen ist, hat er sich bisher unserem näheren Kenntnis entzogen. Wie entsteht denn die Fällung der Salze auf die organische Knochenmatrix und welche Faktoren könnten bei deren Aufnahme mitwirken? Bei der Beurteilung dieses Problems muss man mit dem Zusammenwirken der örtlichen, humoralen, zellularen und allgemeinwirkenden Systemfaktoren rechnen. Die Aufnahme der Knochensalze in die Matrix dürfte der allgemeinen Auffassung nach als vorzugsweise auf die folgenden zwei Weisen ermöglicht vorgestellt werden können.

1) Durch direkte Ausfällung in einem von Salzen übersättigten Medium, wobei das Knochengewebe selbst eine verhältnismässig passive Rolle spielen dürfte.

2) Gemäss der zweiten Theorie besässe die Matrix ein mehr oder minder aktives Vermögen die für die Knochenbildung wichtigen Salze aufzunehmen und zu binden.

Der erste ausführliche experimentelle Beweis für das Bestehen eines quantitativen Verhältnisses zwischen der Verknöcherungsintensität und der Konzentration des Calciums und Phosprohors im Serum ist in den zweien klassischen Arbeiten von HOWLAND und KRAMER (1922) und SHIPLEY, KRAMER und HOWLAND (1926) erbracht worden. Durch Inkubation des Knorpels rachitischer Ratten im Serum und in anorganischen Lösungen ähnlicher Zusammensetzung haben HOWLAND und KRAMER histologisch nachgewiesen, dass eine Calcification der Präparate statt fand. Das histologische Bild zeigte, dass die Verkalkung in vitro genau in derselben Weise wie in vivo vor sich ging. Die Versuche wurden bei einer Temperatur von 37° und einer Inkubationsdauer von 48 Stunden ausgeführt. Eine Vorbedingung der Calcification war, dass der Wert des Formelproduktes Ca × P durchschnittlich 30 mg % übersteigen musste. Diese Zahl entspricht im grossen ganzen dem Wert des Löslichkeitsprodukts für $CaHPO_4$ (SHEAR und KRAMER 1928). Später konnten u. a. ROBISON MC LEOD und ROSENHEIM (1930) in vitro und KRAMER, SHEAR und SIEGEL (1931) in vivo die

Beobachtungen von SHIPLEY bestätigen, indem sie bei ihren Versuchen mit rachitischen Ratten eine Calcification des Knorpels in den Fällen, wo das Produkt Ca × P in Inkubationslösungen oder im Serum 35—40 mg % unterstieg, histologisch nicht nachweisen konnten.

HOLT, LA MERE und CROWN (1925) gaben Anregung zu der Annahme, dass Phosphat, Calcium und Carbonat sich in dem Plasma und in der interstitiellen, die Knochenmatrix umspülenden Lymphe gewöhnlich im Überschuss befinden. Bei ihren Experimenten haben sie wahrgenommen, dass das Löslichkeitsprodukt $(Ca^{++})^3 \times (PO^{\equiv})^2$ in dem Plasma Werte aufzuweisen hatte, welche zeigten, dass das Plasma unter normalen Verhältnissen bis zu einem Werte von 200 % mit $Ca_3(PO_4)_2$ übersättigt wäre, was die Ausfällung des Salzes in die Matrix ermöglichen solle.

Klement und *Weber* (1941) haben neulich geltend gemacht, dass das Serum gewöhnlich mit Hydroxylapatit übersättigt ist. Da dieses Salz nach denselben Forschern auch den Haptbestandteil der anorganischen Knochensubstanz ausmacht, dürfte eine solche Übersättigung von grosser Bedeutung für die Ossifikation sein.

Die Hypothese, dass Calcium und Phosphor in normalem Plasma sich im Überschuss befinden, ist jedoch von einigen Forschern in Zweifel gezogen worden. LOGAN und TAYLOR (1937—38) haben dieses Problem durch Präzipitationsversuche unter Zusatz von Calciumchlorid zu einer Phosphat enthaltenden Lösung bei einem pH von 7.4 beleuchtet und als erstes Fällungsprodukt dabei $Ca_3(PO_4)_2$ erhalten. Es erwies sich jedoch, dass das Löslichkeitsprodukt dieses Salzes in normalem Plasma mit dem Ionenprodukte übereinstimmt, welches darauf deutet, dass das Plasma unter normalen Verhältnissen mit Calcium und Phosphor nicht in dem Sinne übersättigt sein dürfte, dass eine spontane Fällung stattfinden könnte. LOGAN und TAYLOR meinen jedoch, dass eine Übersättigung des Plasma mit Salzen in dem Augenblicke, wo es dieselben an die Matrix abgiebt, die Voraussetzung der Fällung sein muss. Wie kommt denn eine solche Übersättigung zu stande?

Um dieses Problem zu vereinfachen, nimmt ROBISON an, dass die Calzification durch eine primäre Fällung von $Ca_3(PO_4)_2$ getätigt wird, wobei die Ionen $PO_4^{\equiv}$ und Ca^{++} sich in der die Matrix umspülenden Lymphe befinden. Die Fällung solle auf Grund des Massenwirkungsgesetzes vorsichgehen, welches für eine Tricalciumphosphat enthaltende Lösung durch die Formel $(Ca^{++})^3 \times (PO_4^{\equiv})^2 = Ca_3(PO_4)_2 = S$ = Löslichkeitsprodukt ausgedruckt werden kann. Das Löslichkeits-Produkt ist konstant bei konstanter Temperatur und konstantem Druck. Eine Erhöhung des Komponenten $PO_4^{\equiv}$ oder Ca^{++} ruft zur Beibehaltung des Gleichgewichtes eine Fällung von $Ca_3(PO_4)_2$ hervor. Es frägt sich, auf welche Weise diese Erhöhung der Ionenkomponenten erzielt werden kann? ROBISON mit seinen Mitarbeitern FELL, KAY, ROSENHEIM, MC LEOD und SOAMES haben in den Jahren 1924—34 bei ihren Versuchen in vitro diese Frage durch die Entdeckung der Phosphatase-Enzyme in

bahnbrechender Weise beleuchtet. Die Grundzüge dieser Experimente sind in Kürze wie folgt: Dünne Scheiben des Epiphysenknorpels von Ratten, welche während 3—4 Wochen auf rachitogener Kost gehalten worden waren, wurden in Calcium und Phosphor enthaltende Salzlösungen eingetaucht. Nach einer Inkubationsdauer von 3—20 Stunden wurde die Calcification des Knorpels einer histologischen Untersuchung unterworfen. Die Grundlösung enthielt NaCl, $NaHCO_3$, KCl und $MgSO_4$ in demselben Molarverhältnis wie in dem Blutplasma. Dieser Lösung wurden Calcium in der Form von $CaCl_2$, anorganischer Phosphor als $NaKHPO_4$, sowie organischer Phosphor in der Form von α- und β-Glycerophosphat in variierenden Mengen (2—50 mg %) beigefügt.

Diese Versuche zeigten die Bedeutung der Phosphorestern für die Calcification, denn sie ergaben, dass bei der in mit anorganischen Knochen-Salzen übersättigten Inkubationslösungen vorsichgehenden Calcification eine Mineralisierung des Knorpels bei bedeutend niedrigeren Werten des Produkts Ca × P beobachtet werden konnte in dem Falle, wo Phosphor in organisch gebundener Form vorlag. Robison ist der Annahme, dass die Calcification des Knorpels durch Zusammenwirken zweier Faktoren hervorgerufen wird. Der eine derselben, der Phosphatasemechanismus setzt voraus, dass das Blut mit Calcium und Phosphor gewöhnlich nicht übersättigt ist, und dass eine Übersättigung — die theoretische Vorbedingung für die Ausfällung der Knochensalze — durch ein »bone enzyme» Phosphatase bewirkt wird, welches aus den Phosphorsäureestern, die vom Blute der Knochenmatrix zugeführt werden, Phosphat zu spalten vermag. »The second inorganic mechanism» tritt in Wirkung nur in mit Calcium und Phosphor übersättigten Lösungen und ermöglicht die Aufnahme dieser Stoffe in die Knochen. Nach Robison sind diese beiden Mechanismen im Knochen von einander abhängig, aber wirken als gesonderte Komponenten in einem und demselben System. Der anorganische Mechanismus hat sich mehr labil als der Phosphatasemechanismus erwiesen. Bei seinen Experimenten konnte nämlich Robison beobachten, dass in anorganischen Phosphor enthaltenden Lösungen beim Vorkommen von Stoffen wie Chloroform, NaCN und NaF, sowie beim Austrocknen der Knorpelpräparate, der zweite Mechanismus versagte und keine Calcification des Knorpels beobachtet werden konnte. Es erwies sich dagegen, dass beim Vorkommen in der Inkubationslösung von Phosphorsäureestern sogar in so geringen Mengen wie nur 0.5 mg % die erwähnte Agenzien keine nennenswerte Wirkung auf den Phosphatasemechanismus ausübten.

Der Phosphatasemechanismus ist nach Robison der wichtigste Faktor der Calcification, denn nach der Lähmung des genannten Mechanismus durch solche Stoffe wie Phenol, NH.OH und Formaldehyd, sowie nach vorheriger Erhitzung des Knorpelpräparats bis auf 100°, konnte keine Calcification durch Einwirkung des nur anorganischen Mechanismus nachgewiesen werden. Ausserdem erwies es sich bei ähnlichen Versuchen in vitro, dass andere Organe, wie z. B. die Niere, eine hohe Phosphataseaktivi-

tät aufweisen können, aber ohne »second mechanism» sind. Jedoch zeigte kein Gewebe sich einen aktiven zweiten Mechanismus ohne den ersten zu besitzen.

Nach ROBISON sollen diese Mechanismen von enzymatischem Charakter sein. Die Phosphataseaktivität konnte quantitativ durch Beobachtung der Fähigkeit der Enzyme Glycerophosphat in Inkubationslösungen zu spalten studiert werden. Die Phosphatasen haben sich als austrocknungswiderständig erwiesen. MAI (1928) hat feststellen können, dass in vitro Phosphorsäure-Estern stärker vom wachsenden als vom fertig ausgebildeten Knochen gespaltet werden. Zahlreiche Verfasser, ULLRICH (1929) unter ihnen, haben bemerkt, dass die Phosphatasetätigkeit bei der Neubildung von Knochen im Zellengewebe erhöht ist, was die Rolle der Phosphatasen bei der Neubildung von Knochengewebe erleuchtet. Bei von ROBISON und ROSENHEIM (1934) angestellten Versuchen hat der anorganische Mechanismus sich als nicht spezifisch für Aufnahme von Calcium und Phosphor in den Knorpel erwiesen, denn ihre Versuche haben dargelegt, dass auch andere Mineralien, wie beispielsweise Ba, Sr, und Mg, in den Knorpel aufgenommen wurden wenn sie in der Inkubationslösung sich im Überschusse befanden.

ROBISON rechnet mit einem »local factor», der die beiden Mechanismen für das Knochengewebe spezifisch machen dürfte. Sollte dieses nicht der Fall sein, wäre es schwierig zu erklären, weshalb auch andere Organe unter normalen Verhältnissen der Verkalkung nicht unterworfen sind.

HOFMEISTER (1910) hat die Hypothese aufgestellt, dass Veränderungen im Kohlensäuregehalt des Blutes und des Knochengewebes auf die Calcification einwirken, und er stützte seine Annahme auf die Beobachtung, dass CO_2 im Überschuss in der Ringer-Lösung $Ca_3(PO_4)_2$ aufzulösen vermag. Eine Verminderung des Kohlensäuregehaltes, was eine Erhöhung der Alkalität bedeutet, dürfte andererseits die Fällung dieses Salzes fördern, welche Theorie späterhin auch von KLEINMANN (1928) u. a. unterstützt worden ist, nach welchem pathologische Verkalkung öfters an den Stellen des Organismus, die eine alkalische Reaktion ergeben, auftreten soll.

Die Hypothese von HOFMEISTER ist theoretisch annehmbar, jedoch ist es schwierig sich vorzustellen, dass der Mechanismus der Ossifikation in der Tat so einfach beschaffen wäre. Man hat freilich keine Ursache die Bedeutung der Kohlensäure bei der Ossifikation zu ignorieren, insbesondere in dem Falle, wo das anfänglich ausfallende Mineralsalz ein Carbonat ist (POLICARD und ROCHE 1937). Die Schwierigkeit in der Beurteilung der von pH bei der Ossification gespielten Rolle rührt von der Schwierigkeit her, welche die intravitale Bestimmung dieses Wertes bietet. ROUS (1925) hat eine Methode hierfür vorgeschlagen, bei der er Phtaleinderivaten in die Venen von Kaninchen einspritzte und die Farbe nachträglich in den verschiedenen Organen im Mikroskop beobachtete. Mit dieser Methode wurde im Blutplasma pH als 7.38 bestimmt. Alle Gewebe hatten eine Reaktion aufzuweisen, die saurer als die des Plasma war. Das Knorpel-Gewebe hatte ein pH von 7.2 oder etwas darunter.

Die Bedeutung von pH beim Verknöcherungsprozess wie es in der Tat vor sich geht, entzieht sich jedoch unserer Kenntnis. Dazu müssten Bestimmungsmethoden vorliegen, die Studien von pH an Ort und Stelle, also in der Matrix selbst, und im intakten Knochengewebe ermöglichen würden. Bei ihren Verknöcherungsexperimenten in vitro haben ROBINSON und ROSENHEIM (1934) dargelegt, dass mässige Veränderungen des Wertes von pH in der Inkubationslösung auf die Intensität der Calcification keine Wirkung ausüben, und sie haben keinen Unterschied im histologischen Bilde des Knorpels nach Inkubation in Lösungen mit einem zwischen 5.4 und 8.9 schwankenden Werte des pH beobachten können. Erst nachdem dieser Wert unter 5, oder über 10 war, konnte eine verminderte Intensität der Verkalkung wahrgenommen werden.

Seit lange hat eine Reihe von Forschern mit der Möglichkeit gerechnet, dass die Ossifikation den Ausdruck eines mehr oder minder aktiven Vermögens der Knochenzellen die für die Knochenbildung wichtigen Salze aufzunehmen darstelle. Diese Cellulartheorie ist von PFAUNDLER (1904) aufgestellt worden und sie ist auf die folgende Wahrnehmung gegründet: Beim Digerieren von feinverteilter Knorpel- und Knochensubstanz in Lösungen, die Calcium in reichlicher Menge (etwa 500 mg %) in der Form von $CaCl_2$ enthielten, bemerkte er nach einer Zeit von 4—24 Stunden durch gravimetrische Bestimmungen eine Verminderung der Calciummenge im Filtrat der Inkubationsmasse. In manchen Fällen hatte diese Abnahme der Kalk-Menge Werte von bis auf 50 % aufzuweisen. Eine entsprechende Veränderung in der Cl-Konzentration konnte dagegen nicht verspürt werden. Gelatine zeigte sich ebenso wie der Knochen fähig Calcium aus Inkubations-Lösungen aufzunehmen. PFAUNDLER hat seine Ergebnisse in der folgenden Hypothese zusammengefasst: »Ein anscheinend von den Knochenzellen ausgehender formativer Reiz verursacht eine fortschreitende Umwandlung eines Bestandteiles des umgebenden Gewebes, wodurch dieses eine spezifische Affinität zu Kalksalzen des Blutes gewinnt. Die derart zum »Kalk-Salzfänger» umgewandelte Masse wird von gelösten Kalksalzmassen durchdrungen, die mit der organischen Grundlage in Verbindung treten».

WELLS (1906) hat dargelegt, dass diese Affinität zum Calcium besonders kennzeichnend für Knorpel ist. Bei Versuchen, in denen er verschiedene Gewebe in die Bauchhöhle von Kaninchen einimpfte, hat es sich bei der nachfolgenden Analyse der Organ-Asche erwiesen, dass das Knorpelgewebe bedeutend mehr Calcium als die anderen Organe aufgenommen hatte.

Diese Untersuchungen, jetzt schon veraltet, sind von grundlegender Bedeutung gewesen, da sie den Ossifikationsmechanismus von einem neuen Gesichtswinkel aus beleuchteten.

In ihren eingehenden, ebenfalls in vitro ausgeführten Experimenten haben FREUDENBERG und GYÖRGY (1920—23) diese »elektive» Adsorptionstheorie entwickelt. Sie inkubierten verschiedenen Tieren entnommenen, vor dem Versuche auf Calcium und Phosphat analysierten Knorpel in fein verteilter Form in $CaCl_2$ enthaltenden Lösungen während einer Zeit von 5 Tagen,

wonach die Knorpelmasse getrocknet und ein Teil derselben auf Calcium analysiert wurde, wobei eine Aufnahme festgestellt werden konnte. Der Rest der Knorpelmasse wurde während 3 Tage mit einer anorganisches Phosphat enthaltenden Lösung nachbehandelt. Nach abgeschlossener Inkubation war der Knorpel erhärtet und erinnerte an Knochensubstanz. Eine neue Analyse zeigte, dass eine Aufnahme von Phosphat stattgefunden und dass auch die Calciummenge sich noch etwas erhöht hatte. Wurde dagegen die Reihenfolge umgeworfen, so dass die Knorpelmasse zuerst mit Phosphat behandelt und nachher in der Calciumlösung inkubiert wurde, fand keine Aufnahme von Phosphat statt. Ebenso negativ fiel das Ergebnis aus, wenn die Knorpelmasse vor der Behandlung mit Phosphat in eine Natriumchloridlösung getaucht wurde. Nach FREUDENBERG und GYÖRGY diente dieses Experiment als Beweis dafür, dass die primäre Anreicherung des Knorpels mit Calcium eine Vorbedingung der Aufnahme von Phosphat war, welche Annahme in der Beobachtung, dass die aufgenommene Phosphatmenge von der primär adsorbierten Calciummenge abhängig war, eine Stütze fand. Es erwies sich, dass auch Magnesium gleich Calcium von Knochen primär adsorbiert werden konnte, was eine nachfolgende Aufnahme von Phosphat ermöglichte. Bei der Verknöcherung des Knorpels scheint eine einfache mechanische Imprägnierung der Matrix mit Calcium und Phosphor nicht statt zu finden, da die Reihenfolge des Experiments im solchen Falle als belanglos angesehen werden könnte. Ausserdem lag die Beobachtung vor, dass, falls pH in der Inkubationslösung 4.8 unterstieg, keine Calcification des Knorpels vor sich ging, was den Gedanken darauf leitet, dass ein vitaler Prozess der Ossifikation zugrunde liegen dürfte.

Auf grund ihrer Untersuchungen stellten FREUNDENBERG und GYÖRGY eine auch von dem Gesichtspunkte interessante Theorie auf, dass sie die Aufnahme von Calcium in die Matrix als einen chemischen Prozess ansehen. Dieser Theorie nach soll die Calcification 3 Phasen umfassen. Während der ersten Phase wird das im Blutplasma und in der Gewebeflüssigkeit aufgelöste Calcium an das Eiweiss der Knorpelmatrix nach folgender vereinfachten Formel adsorbiert:

I. Calcium + Knorpelprotein = Calciumprotein.

In der zweiten Phase nimmt diese Verbindung Phosphate und Carbonate auf, welcher Vorgang wie folgt bezeichnet werden kann:

II. a) Calciumprotein + Phosphat = Calciumproteinphosphat
b) Calciumprotein + Carbonat = Calciumproteincarbonat,

Der Mechanismus scheint in dieser Phase eine experimentelle Stütze in der Beobachtung zu finden, dass Calcium von Knorpel in der Gegenwart von Phosphat oder Carbonat besser aufgenommen wurde. Da jedoch der Mineraliengehalt des ausgebildeten Knochens bedeutend höher ist als die Eiweissmenge in der Grundsubstanz, erachten FREUDENBERG und GYÖRGY es für nötig noch eine dritte Phase im Ossifikationsmechanismus zu unterscheiden, in der das gebildete labile Calciumproteinphosphat bzw. Carbo-

natkomplex zerfällt und dabei Eiweiss, welches wiederum als »Kalkfänger» dienen könne, freimachen würde. Die Reaktion solle also lauten:

III. a) Calciumproteinphosphat = Protein + Calciumphosphat
b) Calciumproteincarbonat = Protein + Calciumcarbonat.

Diese dritte Phase ist jedoch durch Experimente nicht bestätigt worden. FREUDENBERG und GYÖRGY rechnen mit der Möglichkeit, dass die Alkalität des Knorpels in dieser Phase zunimmt, was seinerseits die Fällung von $Ca_3(PO_4)_2$ befördern würde.

Beurteilt man die Beobachtungen von ROBISON im Lichte der von FREUDENBERG und GYÖRGY aufgestellten Ossifikationstheorie, so dürfte der »Phosphatasemechanismus» bei der Aufnahme von Phosphor in die Knochen seine besondere Geltung in der zweiten Phase haben. Die Annahme, dass die Knochenbildung als ein Ausdruck eines aktiven Prozesses angesehen werden kann, wird auch von der obenerwähnten Beobachtung ROBISONS unterstützt, nach welcher die Calcification in vitro von Zellengiften gelähmt wird.

Das Wesentliche der Ossificationstheorie von FREUDENBERG und GYÖRGY liegt also darin, dass die Verknöcherung durch eine primäre Aufnahme von Calcium gekennzeichnet wird, und diese Annahme ist späterhin immermehr bestätigt worden (ULLRICH 1929, HESS 1930 u.a.). Aus vorwiegend theoretischen Gründen hält in der letzten Zeit LOGAN (1940) es für wahrscheinlich, dass die einleitende Phase der Ossifikation vielleicht eine gleichzeitige Ausfällung von Calcium- und Phosphat-Ionen in einem annähernden Verhältnis von 1:1 sei.

Einen gewissen Beleg zur Annahme, dass die Eigenschaft von »Kalk-Fänger» für das Knochengewebe charakteristisch wäre, bietet eine Forschungsrichtung, die mit dem Vorhandensein eines spezifischen osteogenetischen Prinzips im Knochen rechnet, welches Problem zuerst von LEVANDER (1938) und später von ANNERSTEN (1940) beleuchtet worden ist. Es ist ihnen gelungen bei der Extraktion von normalem Knochengewebe mit Alkohol eine Substanz zu gewinnen, die in die Muskeln von Kaninchen injiziert, Knorpel- und Knochenbildung hervorruf. Dieser osteogenetische Faktor hat sich weder individual- noch artspezifisch erwiesen und wird durch Erhitzung auf 120° inaktiviert.

Die obenerwähnten Hypothesen, von denen eine jede den Mechanismus der Knochenbildung zu erklären versucht, geben uns keine Möglichkeit ein einheitliches Schema, das die Ossifikation in allen ihren Phasen deuten könnte, aufzustellen. Die Ergebnisse zeigen, im Gegenteil, zu genüge, wie viele Lücken unser Wissen aufzuweisen hat. Die Knochenbildung darf zwar allem Anschein nach nicht als eine lediglich isolierte Erscheinung im lebenden Organismus betrachtet werden können. Sie ist eher ein Ergebnis des Zusammenwirkens verschiedener Prozesse in einem grossen System, worin die Mitwirkung aller dieser Prozesse eine Voraussetzung des normalen Verlaufs der Knochenbildung ausmacht. In ihrer Eigenschaft eines

vitalen Prozesses steht die Knochenbildung unter dem Einflusse vieler allgemeinwirkenden Faktoren, welche die Lebensfunktionen im Organismus überwachen und regeln. Von diesen Faktoren kommen in erster Reihe in Betracht Vitamine und Hormone, welche die Knochengestaltung entweder direkt oder indirekt beeinflussen können. Von diesen die Ossifikation beeinflussenden Faktoren kann nur die vom Vitamin D gespielte Rolle im Ramen der vorliegenden Arbeit erörtert werden.

B. Vitamin D und Knochenbildung.

Die von der Rachitis hervorgerufenen pathologischen Veränderungen im Skelett sind schon seit lange bekannt. Mit der Entdetkung des Vitamins D vor etwa 25 Jahren hat die Frage von der Ätiologie der Rachitis, welches Problem die Forschung seit der Zeit von Glisson beschäftigt hat, im grossen ganzen ihren Abschluss gefunden. Nunmehr wissen wir, dass die Rachitis eine Stoffwechselkrankheit des Wachstumsalters ist, die ihren wesentlichen Grund in einer D-Avitaminose hat. Der heilende Einfluss des Vitamins D, was dessen Schlusseffekt betrifft, ist genau untersucht und bekannt. Er äussert sich in einer Aufhebung der bei dieser Krankheit auftretenden mittelbaren und unmittelbaren Störungen, deren hauptsächliche klinische Äusserung die mangelhafte Mineralisierung des Skeletts ist. Die unvollständige Verknöcherung ist die Folge einer Störung im Calcium- und Phosphormetabolismus des Organismus, die für den mangelhaften Mineral-Stoffwechsel in dieser Krankheit kennzeichnend ist. Soll man jedoch die Frage vom physiologischen Wirkungsmechanismus beantworten, oder eher auf das Problem eingehen, *wo* das D-Vitamin im Organismus primär eingreift, so können wir heute nur von Hypothesen aber nicht von sicherer Gewissheit sprechen. Heymann (1938) beleuchtet dieses wie folgt: »The only fact, that can be stated with any degree of certainty, concerning the mode of Vitamin D, is that vitamin D cures rickets by readjusting the disturbed phosphorus and calcium metabolism».

Verschiedene Beobachtungen über den heilenden Einfluss des D-Vitamins auf Rachitis haben jedoch viele wertvolle Einzelheiten für das Aus-Gründen des »Aktionsmodus» des D-Vitamins ergeben, die es uns jedoch infolge der Lücken in unserem Wissen, zu einem einheitlichen System zusammenzustellen noch nicht vergönnt worden ist. Die Literatur hat somit eine grosse Anzahl verschiedener Theorien betreffend den Wirkungsmechanismus des D-Vitamins aufzuweisen, in denen die Ansichten in wesentlichen Punkten auseinandergehen.

Die Schwierigkeiten in der Beurteilung der vom Vitamin D in der Knochenbildung gespielten Rolle müssen unter Berücksichtigung unserer mangelhaften Kenntnisse der in der normalen Ossifikation teilnehmenden Prozesse betrachtet werden.

1. *Die Zusammensetzung der Knochen bei Rachitis.*

Für rachitische Knochen und Knorpel ist eine Verminderung des Mineralienbestandes kennzeichnend. Unter normalen Verhältnissen enthält die Knochensubstanz des Menschen 50—60 % Trockensubstanz, wogegen dieser Wert bei Rachitis nur 20—30 % ist (HARRIS 1935). Bei an rachitischen Ratten ausgeführten Versuchen haben HESS, BERLINER und WEINSTOCK (1931) dargelegt, dass die Abnahme in der Aschenmenge des Femur hauptsächlich in den Metaphysen zum Vorschein kommt, in welchen nach der Heilung des Rachitis auch die grösste Hinzukunft von Mineralien beobachtet werden kann. SCHABAD (1909) gibt uns die folgenden Angaben über den prozentualen Mineralienbestand im rachitischen und normalen Knorpel von Kindern:

	Normaler Knorpel	Rachitischer Knorpel
CaO	21.75	16.06
P_2O_5	16.85	12.52
Andere Salze	2.43	2.71

Die Abnahme in dem Mineraliengehalt der rachitischen Knochen-Substanz macht sich hauptsächlich für Calcium, Phosphor und Carbonat geltend. Die Magnesiummenge ist dagegen relativ erhöht. Der Gehalt von Alkalimetallen und Chlor ist in normalen und rachitischen Knochen annäherend gleich. Diese Veränderungen den normalen Verhältnissen gegenüber werden im allgemeinen als kennzeichnend für die rachitische Knochen- und Knorpelsubstanz angesehen.

2. *Über den Entstehungsmechanismus der rachitischen Knochenveränderungen.*

Wie entsteht denn die mangelhafte Mineralisierung des Skeletts bei Rachitis und wo ist die primäre Störung zu suchen? — Bei der Beurteilung dieser Frage darf man sowohl mit humoralen, intestinalen und örtlich zellularen, wie auch mit allgemeinen Kausalmomenten rechnen. Den verschiedenen Theorien nach soll die Rolle des D-Vitamins in der Heilung rachitischer Skelettveränderungen in erster Reihe in der Aufhebung der dieselben hervorrufenden primären Ursache bestehen.

a) *Die humorale Theorie.* Eine Abnahme in der Kalk- und Phosphor-Retention ist für Störungen im Mineralstoffwechsel bei Rachitis kennzeichnend, was im verminderten Bestand dieser Komponente im Blute zum Ausdruck kommt. IVERSEN und LENSTRUP (1919) sowie HOWLAND und KRAMER (1921) waren die ersten die eine Senkung des anorganischen Phosphorgehaltes im Blute bei Rachitis bemerkten, und diese Veränderung wurde nachträglich als pathognomonisch für diese Krankheit festgestellt. GYÖRGY (1926) hat die Menge der gesamten säurelöslichen Phosphorfraktion im

Blute bestimmt und keinen Zusammenhang zwischen dieser und der Intensität von Rachitis nachweisen können. Späterhin hat jedoch eine Reihe von Forschern wahrnehmen können, dass zusammen mit der anorganischen Phosphorfraktion auch der Gehalt an estergebundenem Phosphor im Blute bei Rachitis vermindert ist (STARE und ELVEHJEM 1932, STEARNS und WARWEG 1935 und BAKWIN, BODANSKY und TURNER 1937). Ganz neulich haben GÖBELL und JANDA (1942) mitgeteilt, dass die Hypophosphatämie bei Rachtitis nur den Phosphagenphosphor berühre, während der »wahre anorganische Phosphor» sowohl bei Rachitis, wie auch beim Zufuhr von Vitamin D im Verhältnis zum normalen Werte einigermassen beständig sein soll. Diese Forscher schreiben dem D-Vitamin die Bedeutung zu, die Phosphagensynthese aus estergebundenem Phosphor befördern zu können. Diese Untersuchungen dürften jedoch auf ihre Bestätigung durch Nachprüfungen warten.

Der allgemeinen Ansicht nach sinkt bei unkomplizierter Rachtis der Calciumgehalt des Serums später als der des Phosphors. (HOWLAND und KRAMER 1921, ROMINGER, MEYER und BOMSKOW 1931, 1934). Die letztgenannten Forscher studierten den Mineralstoffwechsel bei rachitischen Kindern und behaupten, dass eine verminderte Phosphor-Retention die primäre Äusserung der Rachitis sei, und dass die Abnahme des Calciums im Serum erst gleichzeitig mit den klinischen und röntgenologischen Symptomen dieser Krankheit beobachtet werden könnten. Nach ROMINGER (1939) soll das Vitamin D dementsprechend zuerst die negative Phosphorbilanz ausgleichen, wogegen die Calciumbilanz erst später positiv werde. Betreffend die Abnahme des Serumkalkes hat HERLIZ (1942) eine kurze Mitteilung veröffentlicht, nach welcher diese Reduktion vorzugsweise der proteingebundenen Calciumfraktion gelten dürfte, die Menge des ionisierten Calciums dagegen im Verhältnis zu dessen Konzentration im normalen Serum in keinem nennenswerten Masse sich verringern würde.

HOWLAND und KRAMER (1921) sind die ersten, die die mangelhafte Ossifikation bei Rachitis als eine Folge der bei dieser Krankheit auftretenden Hypophosphatämie und Hypocalcämie betrachten. Eine Unterstützung dieser Annahme hat SHIPLEY (1924) erbracht, der die histologische Beobachtung machte, dass Knochen rachitischer Ratten in normalem, aber nicht in rachitischem Serum, calcifiziert wurden. CROXATTO (1931) u. a. bestätigte dieses Ergebnis durch Kultivierung von Hühner-Embryoknochen im Serum gesunder und rachitischer Kinder.

SHIPLEY, KRAMER und HOWLAND (1925, 1926), und ROBISON und Mit-Arbeiter (1926—34) sowie MEYER ZU HÖRSTE (1931) haben vermittelst histologischer Versuche in vitro dargelegt, dass eine der Bedingungen für die Calcification ist, dass der Wert für Ca × P in der Inkubationslösung 30—40 mg % übersteigt, und dass der Umstand, dass Knorpel in rachitischem Serum nicht calcifiziert wird, darauf zurückzuführen ist, dass im letzteren das Produkt Ca × P unter diesem Werte liegt. KRAMER, SHEAR und SIEGEL (1931) haben bei entsprechenden Versuchen in vivo eine

Calcifikation von Knochen rachitischer Ratten beobachtet, sobald Ca × P im Blutserum 40 mg % übersteigt.

BENJAMIN und HESS (1933), die dem Calciumphosphorkomplex eine grosse Rolle bei der Ossifikation in seiner Eigenschaft eines leicht adsorbierbaren Substrats zuschreiben, behaupten, dass die Menge dieses Komplexes bei Rachitis verringert sei, worin sie einen Grund der mangelhaften Ossifikation bei dieser Krankheit ersehen. ROMINGER (1939) verteidigt den Standpunkt, dass die wesentliche Störung bei Rachitis auf den Mangel an einem Ferment, der die Umbildung des Phosphors im Blute in eine die Aufnahme desselben in die Knochensubstanz ermöglichende Form aktiviert, zurückzuführen sei, und nach ihm soll das Vitamin D die Rolle eines solchen Ferments spielen.

Nach der obenerwähnten »humoralen» Theorie dürfte der primäre Anlass der mangelhaften Knochenbildung bei Rachitis in einer quantitativ oder qualitativ mangelhaften Zusammensetzung der Knochenmineralien im Blute zu suchen sein. Dagegen besitze der Knochen selbst bei Rachitis alle Voraussetzungen zur Calcifikation. Die Verknöcherungsstörungen bei Rachitis müssten deshalb als eine »disease of blood and not of the bone» aufgefasst werden (HOWLAND und KRAMER 1921).

b) *Die Adsorptions- und Zellular-sowie andere theorien.* Die mangelhafte Resorbtion von Calcium und Phosphor im Darme bei Rachitis ist seit den grundlegenden Untersuchungen von FINDLAY (1924) und TELFER (1926) bekannt. Demgemäss wird die Beobachtung, dass die Zuführung von Vitamin D den verminderten Calcium- und Phosphorgehalt im Blute bei Rachitis auf normale Werte zurückführt, im allgemeinen als eine Äusserung der vom Vitamin D auf den intestinalen Calcium- oder Phosphor-Stoffwechsel direkt oder indirekt ausgeübten stimulierenden Wirkung aufgefasst (SMITH und SPECTATOR 1940). Die Frage, ob das D-Vitamin dabei eine primäre Wirkung auf den Calcium- oder Phosphormetabolismus ausübt, hat die Forschung seit lange beschäftigt. BERGHEIM (1926) u. a. rechnet mit der Möglichkeit, das das D-Vitamin die Adsorption von Calcium und Phosphor im Darme primär erhöht, und er gründet diese Annahme auf Analysen der Darmschleimhaut rachitischer Ratten auf Calcium und Phosphor bei Zuführung von Vitamin D. PEOLA und GUASSARDO (1931) haben die Resorption von Calcium und Phosphor im Darme rachitischer Hunde untersucht und sind zu dem Ergebnis gekommen, dass die Resorption von Calcium bei Rachitis unverändert bleibe, wogegen die Aufnahme von Phosphor in einer späteren Stufe der Krankheit einen Rückgang aufweise.

In der letzten Zeit hat NICOLAYSEN (1936—1937) die Rolle des Vitamins D bei der Aufnahme von Calcium und Phosphor in isolierten Dünndarm-Schlingen rachitischer Ratten vermittelst ausführlicher Experimente studiert. Die Aufnahme von Phosphor in anorganischer und organischer Form bei normalen und rachitischen Tieren erwies sich bei Mangel an Calcium im Futter als gleich stark. Beim Hunger an Phosphor dage-

gen wurde eine zugeführte Calciummenge von 15 mg im Darme normaler Ratten völlig aufgenommen, wogegen rachitische Tiere unter denselben Verhältnissen nur 5.4 mg aufnahmen. In den verschiedenen Versuchen war die aufgenommene Calcium- und Phosphormenge proportional mit dem Gehalte dieser Stoffe im Futter, welche Beobachtung nach NICOLAYSEN dafür spricht, dass die Absorption von Calcium und Phosphor im Darme ein Diffusionsprozess wäre. NICOLAYSEN rechnet damit, dass das Vitamin D eine primäre Wirkung auf den Calciumstoffwechsel im Darme ausübt, wogegen die Veränderungen im Phosphormetabolismus von sekundärer Natur wären.

LASKOWSKI (1937) und GREENBERG (1939) u. a. halten es ebenfalls für wahrscheinlich, dass das Vitamin D eher den Metabolismus von Calcium als den des Phosphors primär erhöhe. Der erstere injizierte Phosphat-Lösungen in ligierte Dünndarmschlingen von Ratten und beobachtete, dass die Zuführung von Calziferol per os die Aufnahme von Phosphor im Darme eher verringerte.

Es ist nicht bekannt ob das D-Vitamin bei der Regulierung des Calcium- und Phosphorstoffwechsels im Darme die Adsorptionspermeabilität der Epithelzellen des Darmes unmittelbar beeinflusse. BOND (1929), NICOLAYSEN (1937) und HEYMANN (1937) u. a. rechnen mit einer solchen Möglichkeit.

HARRIS und INNES (1931), sowie HARRIS (1933—35) haben unsere Aufmerksamkeit darauf gerichtet, dass das Vitamin D auch die Exkretion von Calcium und Phosphor im Darme verringert. Es hat sich nämlich bei Versuchen mit rachitischen Ratten erwiesen, dass dieselben bedeutend mehr Calcium und Phosphor mit den Faecalien als die gesunden verloren (HARRIS 1935). Eine erhöhte Ausscheidung von Phosphat durch den Harn bei Rachitis ist ebenfalls beobachten worden (HEYMANN 1928, u. a). HARRIS und INNES schreiben dem Vitamin D die Bedeutung zu, die »net absorbtion» von Kalk und Phosphat im Darme zu erhöhen, was sich durch eine erhöhte Adsorption und verringerte Exkretion dieser Stoffe in der Anwesenheit von Vitamin D äussert. Die Beobachtung, dass eine aktive Exkretion von Calcium und Phosphor, und dabei hauptsächlich im Colon bestehe, ist später u. a. von COWELL (1937) bestätigt worden.

Gemäss den obenerwähnten Forschungen dürfte die hauptsächliche Aufgabe des Vitamins D bei der Heilung von Rachitis in der Wiederherstellung des intestinalen Kalk- und Phosphorstoffwechsels bestehen, wodurch die Zufuhr von »Bausteinen» an die Knochen im genügenden Ausmasse ermöglicht wird. Der Einfluss des Vitamins D auf die Ossifikation selbst dürfte unter diesen Verhältnissen von sekundärer Natur sein (HARRIS 1935).

Eine gewisse Stütze findet diese Theorie in der Beobachtung, dass beim Mangel an Vitamin D Rachitis bei Ratten durch Verabreichung von phosphorarmer Kost hervorgerufen werden kann. Darauf gründet sich das Prinzip der von MC COLLUM und STEENBOCK vorgeschlagenen gewöhnlichen

rachitogenen Diäten, in welchen das Verhältnis Ca: P gleich 4: 1 bzw. 5: 1 ist. Auf diese Weise wird eine negative Phosphorbilanz geschafft, weil die Exkretion von endogenem Phosphor bei der Zuführung eines Überschusses an Calcium in der Diät sich erhöht (NICOLAYSEN 1937). Eine Folge dieses »P-Opfers» (ROMINGER 1939) ist die Hypophosphatämie, welche ihrerseits zu rachitischen Ossifikationsstörungen führt. Bei an Ratten ausgeführten Experimenten ist es demgemäss gelungen, ohne Mitwirken von Vitamin D Rachitis bloss durch Normalisierung des Verhältnisses Ca: P in der Diät zu heilen (GLANZMANN 1925, KRAMER, SHEAR und SIEGEL 1931, LILLY 1932 u. a.). Neulich haben NICOLAYSEN und JENSEN (1939) dargelegt, dass ein Zusatz von Vitamin D zur rachitogenen Diät bei Ratten den Gehalt an Asche im Femur von 29,9 auf 38.8 % erhöhte, und dass weiterhin eine subcutane Injektion von 12 mg Phosphor pro Tage diesen Wert auf 45.4 % brachte.

Es wäre jedoch hier angebracht, den Unterschied zwischen der bei Versuchstieren hervorgerufenen experimentellen Rachitis und der spontanen Kinderrachitis zu betonen, denn während die erstere mit Hilfe von phosphorarmer Diät hervorgerufen werden kann, scheint dieses Sachverhältnis bei der Entstehung von Rachitis bei Kindern nicht in derselben Weise bestimmend zu sein, da ja diese, wie bekannt, nur durch Zuführung von Phosphor in die Diät sich nicht heilen lässt. Das Verhältnis zwischen Kalk und Phosphor ist in der Muttermilch etwa 1.31, in Kuhmilch aber nur 0.73, weshalb Säuglinge, wenn das Verhältnis dasselbe wie bei Experimenten an Tieren wäre, der Rachitis mehr heimgefallen sein müssten, als die mit Kuhmilch ernährten Kinder. Das Sachverhältnis ist aber, wie bekannt, entgegengesetzt. Rattenrachitis wird andererseits unter Einfluss des Vitamins D geheilt, ohne dass die Quote C : P der rachitogenen Diät verändert werden muss, und denselben heilenden Effekt zeigt das Vitamin D auch bei Kinderrachitis.

Von der Zeit an, wo die Frage von der Pathogenese der Rachitis zum Gegenstand eingehenden Studiums gemacht wurde, hat eine Reihe von Forschern mit der Möglichkeit gerechnet, dass die bei dieser Krankheit auftretenden Ossifikationsstörungen vermutlich auf eine Unfähigkeit der Knochen selbst die für die Knochenbildung wichtigen Salze zu verwerten zurückgeführt werden könnten. Nach dieser Theorie solle die vom Vitamin D gespielte Rolle in der Beseitigung dieses örtlichen Missverhältnisses in der Knochensubstanz bestehen. PFAUNDLER (1904) war als erster der Annahme, dass bei Rachitis die Knochen an einer primären Unfähigkeit sich zu calcifizieren leiden. »Bei Rachitis werden Kalksalzmassen in unzureichender Menge eingelagert oder unzureichend fixiert, weil die spezifische Affinität mangelt.» In Übereinstimmung mit PFAUNDLERS Theorie ist die Umbildung der Zellen des osteoiden rachitischen Knochengewebes in »Kalksalzfänger» eine Voraussetzung für die Normalisierung dieses Verhältnisses. Die Annahme, dass es den Knochen bei Rachitis an einem aktiven Calcifikationsprinzip mangelt, wurde später u. a. von BOSÁNYI

(1925) unterstützt. Es gelang ihm nämlich Rachitis bei Ratten vermittelst Knochenmark gesunder Tiere zu heilen, wogegen rachitischer Knochen-Mark denselben Effekt nicht aufweisen konnte. Eine Reihe der in der letzten Zeit ausgeführten Beobachtungen (VOLLMER 1936 und HEYMANN 1937) zeigen, dass das Vitamin D in fettreichen Geweben, wie beispielsweise Knochenmark, aufgespeichert wird, weshalb das von BOSÁNYI erreichte Ergebnis teilweise auf den Einfluss des Vitamins D zurückgeführt werden kann.

FREUDENBERG und GYÖRGY betrachten die mangelhafte Verknöcherung bei Rachitis im Lichte ihrer Calcificationstheorie, welche bereits im Zusammenhange mit der normalen Ossifikation geschildert worden ist. Nach diesen Forschern soll bei Rachitis der Verknöcherungsvorgang in der einleitenden, von der Adsorbtion des Calciums an die Proteinsubstanz des Knorpelgewebes gekennzeichneten Phase aufhören, und die Bindung von Phosphat könnte darum während der zweiten Ossifikationsphase nicht zu stande kommen.

FREUDENBERG und GYÖRGY rechnen mit einem lokalen »Hemmungs-Mechanismus» als der Ursache der mangelhaften Mineralisierung des Skeletts bei der Rachitis. Im Falle, dass die lokale Reaktion der Knochensubstanz zu sauer ist, was ihrer Annahme nach bei Rachitis der Fall sei, würde dieses eine hemmende Wirkung auf die Ossifikation ausüben. Da Kuhmilch ein saures Milieu hervorruft, dürfte dieselbe eher für Rachitis prädisponieren als Muttermilch, die ein alkalisches Milieu schafft.

ULLRICH (1929) ist der Ansicht, dass der grundsätzliche Unterschied zwischen experimenteller Rachitis bei Tieren und spontaner Rachitis bei Kindern darin liegt, dass bei den letzteren die Ossifikation schon in der einleitenden Phase gestört sein dürfte. Sollte die primäre Anreicherung des Calciums, welche eine Voraussetzung für die nachträgliche Aufnahme von Phosphat ist, in der ersten Phase ausbleiben, würde dieses teilweise erklären, weshalb die Zuführung von nur Phosphat, wie bekannt, bei spontaner Kinderrachitis keinen heilenden Effekt auf die Ossification ausübt.

ROBISON und seine Mithelfer (1923—34) die bei Ihren Versuchen die Bedeutung der Phosphorsäureestern bei der Ossifikation wahrgenommen haben, sind der Annahme, dass die gehemmte Knochenbildung bei Rachitis eine Folge der verringerten Phosphatasetätigkeit in den Knochenzellen ist, was jedoch nähere Untersuchungen nicht bestätigen, da es vielmehr bekannt ist, dass die Phosphatasen in rachitischer Knorpel- und Knochen-Substanz eine erhöhte Wirksamkeit aufweisen (DEMUTH 1925, MAI 1928, CAREDDU und BRIGENTI 1936, KODAMA 1939 u.a.). Die erhöhte Phosphatase-Tätigkeit in den Knochengeweben bei Rachitis muss vielleicht im Lichte der starken Neubildung von Knorpel bei dieser Krankheit, für die eine Erhöhung im Phosphorstoffwechsel kennzeichnend ist, betrachtet werden (DOLS, JENSEN, SIZOO und VAN DER MAAS 1939). Der »Phosphatasemechanismus» kann jedoch aus irgend einer Ursache im rachitischen

Knorpel nicht zur Geltung kommen. POLICARD, PÉHU und BOUCOMONT (1934) sind der Meinung, dass dies entweder in »humoralen Störungen» infolge eines Mangels an den für die Knochenbildung wichtigen Phosphor-Estern im Blute seinen Anlass hat, oder auch rechnen sie mit der Möglichkeit, dass ein toxischer Prozess die Ausnutzung dieser Estern beim Rachitis stören könne. Eine Bestätigung der ersten Annahme ist unter anderen von STARE und ELVEHJEM (1932) gegeben worden, welche eine Verringerung des Gehaltes von Phosphorsäureestern im Blute gleichzeitig mit anorganischer Hypophosphatämie beobachtet haben. In den Erythrocyten soll bei Rachitis der Phosphorsäureesterngehalt ebenfalls vermindert sein (FREUDENBERG 1935).

Es gibt nur wenige experimentelle, auf Beobachtungen bei der Zuführung von Vitamin angestellte Untersuchungen des vom D-Vitamin ausgeübten unmittelbaren Einflusses auf die Knochensubstanz selbst. ROBISON und ROSENHEIM (1934) haben die Calcification von rachitischem Rattenknorpel nach Zusatz von Ergosterol in die Inkubationslösung, oder bei vorheriger Behandlung des Knorpels mit Ergosterol studiert, wobei dieser Agenz keinen Einfluss auf die Calcification des Knorpels ausgeübt zu haben scheint. Dieses negative Resultat müsse jedoch nach ROBISON mit Zurückhaltung angenommen werden. Etwas später in demselben Jahre teilt ROSENHEIM mit, dass die Calcificationskraft des Knorpels rachitischer Ratten immer mehr sich verminderte, je längere Zeit die Tiere auf rachitogener Diät gehalten wurden. FLEISCHMANN (1937) erklärt ebenfalls im Anschluss an eine kurze histologische Untersuchung in vitro, dass die Calcification von Vogelknochen langsamer in rachitischem als in normalem Serum vor sich geht, und dass ein Zusatz von Calcium oder Phosphat zu der Inkubationslösung den Mangel an Vitamin D nicht vollständig kompensiert. Bei Untersuchungen in vitro ist auch PFEIFFER (1936) zu einem ähnlichen Ergebnis gekommen.

Unter Benutzung der in vitro-Methodik ROBISONS haben VENAR und TODD (1936) in einer kurzen Veröffentlichung Untersuchungen über den Effekt des D-Vitamins auf die Calcification der Tibia von Ratten angestellt und teilen mit, dass sie durch Behandlung von Ergosterol mit Aceton und Talk ein wasserlösliches antirachitisches Prinzip »Aquasterol» gewonnen haben. Ein Zusatz dieses wasserlöslichen D-Vitamins in die Inkubations-Lösung erhöhte die Calcification in der »growing area» der Tibia rachitischer Ratten. Die Intensität der Calcification war bei konstantem D-Vitamin-Gehalt proportional zu der Salzkonzentration der Inkubationslösung.

Es ist von alters her bekannt, dass die pathologischen Veränderungen im Skelett bei Rachitis nicht als eine isolierte Krankheit des Knochen-Gewebes oder als ausschliesslich eine Störung des Mineralstoffwechsels im Organismus zu betrachten sind, und in Übereinstimmung mit dieser Beobachtung erstreckst sich der Effekt des D-Vitamins auch auf die allgemeinen organischen Zellen- und Körper-Funktionen. In diesem Zusammenhange können u. a. die von RÄIHÄ, HELSKE, PEITSARA und VEHNIÄI-

NEN (1937) ausgeführten Untersuchungen erwähnt werden, welche die grosse Bedeutung des D-Vitamins für die im Kohlehydratstoffwechsel einbegriffenen Prozesse darlegen. Diesen Beobachtungen liegen Versuche mit rachitischen Kaninchen zu grunde. Nach RÄIHÄ und seinen Mitarbeitern werden die primären Störungen bei Rachitis von einer mangelhaften Spaltung der organischen Phosphorsäureestern im Organismus, und dabei vorwiegend in den Muskeln, gekennzieichnet. Dieser Überschuss an estergebundenem Phosphor sollte die bei dieser Krankheit vorkommende anorganische Hypophosphatämie verursachen. Wurde den Tieren ein D-Vitamin-Stoss gegeben, zeigte es sich, dass die Muskelkraft sich innerhalb einer kurzen Zeit erhöhte, welches gleichzeitig zu einer Erhöhung der anorganischen Phosphorfraktion im Blute führte. Dadurch sollte ein für die Verknöcherung günstiges Milieu — wahrscheinlich vermittelst der gleichzeitigen Entstehung des sogenannten Hess'schen Komplexes — geschafft werden. Einen weiteren experimentellen Beweis für die Bedeutung der Muskeltätigkeit beim Heilen von Rachitis gibt PEITSARA in einer gegenwärtig im Druck befindlichen Arbeit, in welcher er unter anderem zeigt, dass, wenn die Extremitäten rachitischer Hunde vor Verabreichung des D-Vitamin-Stosses gegipst wurden, keine nennenswerte Veränderungen in den Phosphorfraktionen des Blutes wahrgenommen werden konnten.

3. *D-Hypervitaminose.*

Kurz nach der Entdeckung des D-Vitamins trafen von verschiedenen Seiten Mitteilungen ein, dass dieses Vitamin bei Gabe an Menschen und Tiere toxische Beiwirkungen haben könne. Beispielsweise haben unter den ersten KREITMAR und MOLL (1928) bei der täglichen Zuführung von 2 mg bestrahlten Ergosterins an Mäuse gewisse Krankheitssymptome wie Appetitverlust, Gewichtsabnahme, Erbrechen, Diarrhoe, Somnolenz und Alopecia beobachtet. In den meisten Fällen starben die Tiere nach 2—3 Wochen. Für Meerschweinchen und Katzen war die letale Dosis Ergosterin bedeutend höher (40 bzw. 10 mg). Gleiche pathologische Veränderungen bei D-Hypervitaminose wurden u. a. von HESS (1928) auch bei Menschen beschrieben.

Die Angaben über die Grenzen der toxischen D-Vitamindosen für verschiedene Tiere schwanken bei den verschiedenen Verfassern verhältnismässig stark. Der allgemeinen Ansicht nach dürfte jedoch das Marginal zwischen den therapeutischen und den toxischen Dosen relativ breit sein. LIGHT, MILLER und FREY (1929) geben beispielsweise auf, dass Ratten eine tägliche Gabe von 10,000 Male die heilende Dosis D-Vitamins während sechs Monate harmlos vertragen können. Erst bei einer Menge von 100,000 Mal die kurative Dosis konnten bei den Tieren toxische Symptome beobachtet werden. REED (1937) ist der Ansicht, dass eine langwierige Zuführung von 1000 Mal die therapeutische Dosis D-Vitamins für Ratten völlig gefahrlos ist. Bei Menschen treten schwerere Komplikationen bei der Zu-

führung grosser Mengen D-Vitamins verhältnismässig selten auf. Dieses wird, unter anderem, auch von den Ergebnissen der HARNAPP'schen »Vitamin-Stosstherapie», welche gegenwärtig in der Bekämpfung der Rachitis erfolgreich angewandt wird, bestätigt.

Die durch eine Überdosierung von D-Vitamin in dem Mineralstoffwechsel hervorgerufenen Veränderungen werden von einer Erhöhung des Calcium- und Phosphorgehaltes im Blute gekennzeichnet, welche Beobachtung von mehreren Forschern beschrieben ist. HESS (1928) gibt beispielsweise eine Erhöhung des Serumcalciums in D-hypervitaminotischen Ratten bis auf 18—20 mg % und von Phosphor auf 10 mg % auf. HARRIS und STEWARD (1929) gaben jungen Ratten eine Diät enthaltend 0.1 % bestrahlten Ergosterins, wobei der Serumkalk sich um 25 und der Phosphor um 50 % erhöhten. Bei Omnivoren scheint bei D-Hypervitaminose vorzugsweise eine Erhöhung des Serumkalkes statt zu finden (HARRIS 1931), wogegen bei Herbivoren die Erhöhung in erster Reihe der Phosphorfraktion gelten dürfte (WARKANY 1930). Bei weit vorgeschrittener D-Hypervitaminose ist neben der Hyperphosphatämie und Hypercalcämie eine Auflagerung von Calcium in verschiedenen Organen nachgewiesen worden, welche Beobachtung von SMITH und ELVOVE (1929) zuerst beschrieben worden ist. Von diesen Organen kommen in erster Reihe die Leber, das Herz, die Blutgefässe, die Nieren und die Milz in Betracht.

Es ist nicht näher bekannt, ob die Hypercalcämie und die Hyperphosphatämie bei der D-Hypervitaminose hauptsächlich ein Ergebnis der erhöhten Adsorption im Darme sind, oder ob der Kalk und der Phosphor aus den Mineralvorräten des Körpers, von welchen das Skelett der wichtigste ist, mobilisiert werden. Vielleicht darf man mit einem Zusammenwirken der beiden Möglichkeiten rechnen. ASHFORD (1930) u. a. schreibt der erhöhten Adsorption die grösste Bedeutung zu. Ebenso haben neulich TWEEDY TEMPLETON, PATRAS, MC JUNKIN und NAMARA (1939) gezeigt, dass massive Dosen D-Vitamins (460000 I. E.) die Adsorption von Calcium und Phosphor im Darm von Ratten in grossem Ausmasse erhöhen. Dieselbe Wirkung wurde auch nach vorangegangener Thyreoideoparathyreoidektomie verspürt, eine Beobachtung, die einer früheren, unter anderen auch von HESS vertretenen Theorie, dass die Hypercalcämie bei D-Hypervitaminose die Wirkung des D-Vitamins via Parathyreoidea als Anlass hat, widersprechen dürfte.

Mehrere Forscher haben andererseits gezeigt, dass eine Überdosierung des Vitamins D eine Demineralisierung des Skeletts zur Folge haben kann. WISKOTT (1930) hat beispielsweise eine Verminderung der Trocken-Substanz in Knochen von Ratten beobachtet, denen er grosse Dosen D-Vitamins (0.25—1 mg pro 10 gr des Körpergewichts) verabreicht hat. Diese Verminderung berührte vorwiegend Calcium und Phosphor in der Zuwachszone. Eine Zuführung grosser Dosen Calciums konnte allein diesem Defekt nicht abhelfen.

Eingehende Untersuchungen von BROWN und SHOHL (1930) beleuchten sehr deutlich die verschiedenen Phasen der Skelettveränderungen bei D-Hypervitaminose. Bei diesen Versuchen wurde Ratten bestrahltes Ergosterol in Dosen von 0,01—2 mg, also 100—20000 Mal die kurative Dose, täglich verabreicht. Bei der Zuführung von Ergosterol in einer Menge von bis 0.5 mg wurde die Aufnahme von Calcium und Phosphor in die Knochen stimuliert, was in einer Erhöhung der Trockensubstanzmenge ihren Ausdruck fand. Überstieg jedoch die verabreichte D-Vitaminmenge 0.5 mg, so trat nach dieser initialen Phase eine Demineralisation der Knochen ein, wobei eine negative Calcium- und Phosphorbilanz entstand, die von einer erheblichen Absonderung dieser Stoffe im Harn, sowie von der Calcification der verschiedenen obenangeführten Organe gekennzeichnet wurde. Rachitische Ratten erwiesen sich gegenüber normalen als mehr wiederstandsfähig für grosse Dosen D-Vitamins. Eine negative Calcium- und Phosphorbilanz trat bei diesen Tieren erst bei Dosen von 2 mg D-Vitamins auf.

Es ist noch nicht vollkommen klargestellt worden, ob die bei einer Überdosierung von Vitamin D im Stoffwechsel auftretenden Veränderungen einer reinen Hypervitaminose zugeschrieben werden können, oder ob sie ein Resultat der Einwirkung von im Vitaminpräparate enthaltenen toxischen Nebenprodukten sind. Die letztere Möglichkeit ist bei den ersten Experimenten mit aktiviertem Ergosterin, welches mehr als *ein* aktives Prinzip zu enthalten vermutet werden darf, nicht ausgeschlossen (LOGAN 1940).

Bekanntlich gibt es verschiedene intermediäre Bestrahlungsprodukte von Ergosterin. Eines von diesen, nämlich Dihydrotachysterin oder AT 10 (HOLTZ 1934), hat in der letzten Zeit besonderes Interesse erweckt. Die Wirkung dieses Stoffes erinnert teilweise an die des D-Vitamins. Bei der Verabreichung von AT 10 in möglichst reiner Form per os oder parenteral an Hunde hat SIWE (1935) eine starke Erhöhung des Serumkalkes schon nach 24 Stunden beobachtet. Der Phosphorgehalt im Serum erlitt dagegen, im Gegensatz zum Effekt des Vitamins D, keine Veränderung und dieses ist wahrscheinlich eine der Ursachen dafür, dass AT 10 allein Rachitis zu heilen nicht vermag.

Andererseits hat es sich dagegen erwiesen, dass hochwertige Antirachitica in möglichst reiner Form bei starker Überdosierung toxische Verkalkungen hervorrufen können, was somit ein Ergebnis einer echten D-Hypervitaminose sein dürfte (ROMINGER 1939).

III. Material und Methodik.

A. Versuchstiere.

Das Tiermaterial umfasst 67 Ratten, 32 Meerschweinchen, 15 Kaninchen und ein Kalbembryo. Es wurden insgesamt 536 diesen Tieren entnommene Knorpel- und Knochenpräparate angewandt. Die Verteilung des Versuchsmaterials geht aus den verschiedenen Experimentserien hervor.

Ratten.

Als Versuchstiere dienten vorzugsweise weisse, in einigen Fällen schwarzscheckige Laboratoriumratten und zwar beinahe alle Männchen, deren Gewicht zwischen 30 und 360 g schwankte, (grösstenteils etwa 200 g).

Die Tiere wurden in Käfigen aus Eisendraht mit doppelten Böden aufgezogen. Bei allen Versuchen, mit Ausnahme der Rachitisuntersuchungen, war die Grundkost von folgender Zusammensetzung:

Maismehl	1,300 g
Weizenkleie	400 »
Casein	180 »
Natriumchlorid	10 »
Calciumcarbonat ..	10 »

Diese Bestandsteile wurden unter Zusatz von Wasser zu einem Brei vermischt. Ausserdem wurde den Tieren täglich trockenes Roggenbrot, einen jeden zweiten Tag Milch, und ein Mal in der Woche gekochte Lunge oder Fisch, sowie häufig Mohrrüben verabreicht. Jeder Käfig enthielt eine Pipettenflasche mit destil-

liertem Wasser ad libitum. Der Ernährungszustand und Zuwachs der Tiere war bei der obenangeführten Kost gut. Die Normaltiere wurden ausschliesslich auf dieser Kost gehalten.

Die *D-Hypervitaminisierung* geschah vermittelst Zuführung von Vigantol (Merck) mit Hilfe einer Rekordspritze und eines Nelatonkatheters, welcher als Ventrikelsonde angewandt wurde. (1 ccm Vigantol enthält 0.3 mg in Sesamöl gelösten Vitamin D_2 und entspricht 12000 I.E. D-Vitamin). Die Vitaminisierung wurde während 4—6 Tagen bewirkt und die Tagesdosen schwankten bei den verschiedenen Versuchen zwischen 12000 und 36000 I. E. Vitamin D. Die Kontrolltiere, die im übrigen unter denselben Verhältnissen, aber in anderen Käfigen aufwuchsen, erhielten anstatt Vigantol eine entsprechende Volummenge reines Sesamöls. In den verschiedenen Versuchsserien wurden die Kontroll- und »Vitamin»-Ratten soweit möglich einem und demselben Wurfe entnommen. Irgendwelche klinisch wahrnehmbare toxische Wirkungen des Vitamins D konnten bei diesen Versuchen nicht festgestellt werden.

D-Avitaminose wurde in Dunkelraum bei 3—4 Wochen alten Ratten vermittelst Mc Collums rachitogener Diät Nr. 3143 (Merck) hervorgerufen. Das Gewicht der Tiere am Anfange der Diätzeit schwankte zwischen 30 und 70 g (meistens etwa 40 g). Bei den verschiedenen Versuchen wurden die Ratten, ebenso wie bei den D-Hypervitaminose-Versuchen, in zwei Gruppen eingeteilt, von denen die eine aus Kontrolltieren bestand. Alle Tiere wurden unter im Übrigen gleichen Verhältnissen aufgezogen, jedoch mit dem Unterschiede, dass die Kontrolltiere 4 Tropfen verdünnter Vigantollösung, enthaltend 50 I.E. Vitamin D täglich erhielten. Nach Guassardo (1937) sollen mit rachitogener Kost gefütterte Ratten bei dieser Tagesdosis Vitamin D vollkommen gesund verbleiben. Die Ratten der zweiten Gruppe erhielten eine entsprechende Volummenge reines Sesamöls. Nach Bomskow (1935) sind bei auf der Diät Mc Collums gehaltenen Ratten schon nach einer Woche rachitische Veränderungen in den Metaphysen histologisch nachweisbar. Bei meinen Versuchen schwankte die Zeit der Diät zwischen 7 und 60 Tagen (vorzugsweise 3—4 Wochen). Nach 2—3 Wochen wiesen die Ratten, die nur Sesamöl erhalten hatten, typische klinische Zeichen der Rachitis auf. Im Gegensatz zu diesen Wahrnehmungen bei den an D-Avitaminose leiden-

den Ratten, schienen die Kontrolltiere sich normal zu entwickeln, ohne dass irgendwelche entsprechende krankhafte Erscheinungen bei ihnen beobachtet werden konnten. Am Ende der Versuchszeit wurde eine Anzahl von Röntgenkontrollen vorgenommen, wobei bei den D-avitaminotischen Ratten typische Anzeichen von Rachitis festgestellt werden konnten, wogegen keine solche Symptome bei den Kontrolltieren zu entdecken waren.

Meerschweinchen.

Die zur Anwendung gekommenen Meerschweinchen, alle von männlichem Geschlecht, waren vorzugsweise Tiere im Wachstums-Alter von 330—700 g (meistens 400—500 g) Gewicht. Als Käfige dienten geräumige Holzkisten, deren Böden mit täglich erneuerten Sägespänen bedeckt waren. Die Grundkost war bei sämtlichen Versuchen die gleiche und bestand vorzugsweise aus trockenem Heu und Hafer. Ausserdem erhielten die Tiere jeden Tag ein Stück Steckrübe, sowie drei bis vier Mal in der Woche Mohrrüben. Die Normaltiere wurden nur mit dieser Kost gefüttert.

Die *D-Hypervitaminisierung* geschah durch Zuführung von Vigantol vermittelst einer Pipette. Die täglichen Dosen waren gewöhnlich 36000 I.E. Vitamin D und die Vitaminisierungszeit schwankte zwischen 4 und 14 Tagen. Die Kontrolltiere, welche getrennt gehalten wurden, erhielten eine entsprechende Volum-Menge reines Sesamöls. Irgendwelche krankhafte Symptome wiesen die Tiere bei diesen Versuchen nicht auf.

Da die Erfahrung gezeigt hat, dass Rachitis bei Meerschweinchen schwer hervorzurufen ist, wurden solche Versuche in der vorliegenden Arbeit nicht veranstaltet.

Kaninchen.

Die Kaninchen wurden ausschliesslich für D-Avitaminose-Versuche angewandt. Die Versuchstiere waren 3—4 Wochen alte Kaninchen, deren Gewicht am Anfange der Diätzeit zwischen 290 und 520 g schwankte. Die Tiere wurden in denen der Meerschweinchen ähnlichen Kisten, aber im Dunkel aufgezogen. Die Kontroll-Tiere erhielten vermittelst Pipette 300 I.E. Vitamin D pro Tag,

wogegen den »Rachitistieren» eine entsprechende Volummenge reines Sesamöls verabreicht wurde. Die Dauer der Diätzeit schwankte zwischen 9 und 57 Tagen. Beim Füttern der Kaninchen erwies es sich jedoch, dass sie sich auf die Mc Collum'sche rachitogene Kost »nicht verstanden». Die Tieren beider Gruppen assen schlecht und bei ihnen konnte eine ausgesprochene Dystrophie verspürt werden. Diese Versuchsserie wurde im Herbst ausgeführt. Räihä und seine Mitarbeitern (1937) haben gezeigt, dass der Herbst eine ungünstige Zeit für die Hervorrufung von Rachitis bei Kaninchen ist, da die Widerstandskraft und der Zuwachs der Tiere in dieser Zeit am schlechtesten sind.

Kalbembryo.

Das angewandte Kalbembryo war etwa 5 Monate alt und wurde sogleich nachdem das Muttertier geschlachtet war vom Schlachthof abgeholt. Da unter den gegenwärtig herrschenden Verhältnissen trächtige Kühe äusserst selten geschlachtet werden, war es mir nur dieses eine Exemplar aufzubringen gelungen.

B. Präparierungsmethodik.

Als Versuchsmaterial wurden die langen Knochen der vorderen und hinteren Extremitäten der Versuchstiere angewandt.

Die Präparierungsmethodik war wie folgt: Das Tier wurde durch Decapitation getötet und in unmittelbarem Anschluss daran die verschiedenen Knochen möglichst steril durch vorsichtiges Entfernen der weichen Teile vermittelst Scheere und scharfen Messers sorgfältig hervorpräpariert. Unverzüglich danach — um mit lebendem Knochengewebe bei den Versuchen operieren zu können — wurden die Präparate in eine Inkubationslösung versenkt, deren Menge bei den verschiedenen Versuchen zwischen 4.0 und 25.0 ccm (bei den meisten Versuchen 10.0 ccm) schwankte. Als Gefässe dienten Probierröhren. Der Spiegel der Lösung stand wenigstens 1 cm über dem Präparate. Die Probierröhren wurden hiernach um Verdunstung zu vermeiden luftdicht mit Baumwollpropfen geschlossen und in Gestelle plaziert. Die Inkubation geschah während 24

Stunden bei Zimmertemperatur (18—22° C). Nach Ablauf dieser Zeit wurden die Präparate den Probierröhren entnommen, mit Filtrierpapier abgetrocknet, und auf einer Mikrowage aufgewogen. Vor der Inkubation wurden die Präparate nicht gewogen um Austrocknen zu vermeiden.

Zur Bezeichnung der bei den in dieser Arbeit erwähnten Versuchen angewandten Knochenpräparate sind die folgenden Verkürzungen zur Anwendung gekommen:

Knochen der vorderen Extremitäten:

H bedeutet *Humerus*
RU » *Radius* und *Ulna*

Demgemäss bedeutet *HRU* die langen Knochen der vorderen Extremität in einem Block mit intaktem Ellenbogengelenk.

Knochen der hinteren Extremitäten:

F bedeutet *Femur*
T » *Tibia*

FT bedeutet also Femur und Tibia zusammen in einem Stück mit intaktem Kniegelenk.

C. Inkubationslösungen.

Als Grundlösung für die Inkubation wurde vorzugsweise Robisons Lösung von folgender Zusammensetzung angewandt:

$NaCl$	6.0	ad 1000 aq. dest.
$NaHCO_3$	2.2	
KCl	0.3	
$MgSO_4 . 7H_2O$	0.25	

Bei einigen Versuchen wurde als Grundlösung eine physiologische 8.5 %-ige Natriumchloridlösung, sowie in einer der Serien destilliertes Wasser angewandt.

Die Aufnahme von Calcium wurde bei einem Zusatz von $CaCl_2 . 6H_2O$ zur Grundlösung studiert. (In den meisten Fällen etwa 11 mg % Ca).

Die entsprechenden Phosphoruntersuchungen wurden mit einem Zusatz von anorganischem Phosphorsalz in der Form von $Na_2 HPO_4$ (nach SÖRENSEN) getätigt. Als organisches Phosphor-Salz kam Na-Glycerophosphat (Merck) zur Anwendung. Die Konzentration des Phosphors schwankte zwischen 7 und 50 mg %.

Die Retention von Calcium und Phosphor in den Präparaten wurde in der Basallösung ohne Zusatz dieser Stoffe studiert.

Alle Lösungen waren beim Anfange der Versuche frisch hergestellt und klar. In einigen Fällen konnte am Ende der Versuche eine schwache Opaleszens in den Lösungen infolgedessen, dass eine kleine Menge Eiweiss aus den Präparaten herausdiffundiert war, beobachtet werden. Bei einigen Experimenten mit komplizierteren Lösungen wurde vor der Inkubation eine schwache initiale Fällung vermittelst Filtrierung entfernt.

D. Chemische Bestimmungsmethodik.

Das Arbeitsprinzip der Versuche war auf Grund der während der Inkubation stattgefundenen Veränderungen in der Calcium- und Phosphorkonzentration der Versuchslösungen einen Ausdruck für die Aufnahme bzw. Retention dieser Stoffe in den Präparaten zu finden.

Die Analyse der Inkubationslösungen auf Calcium und Phosphor wurde unverzüglich nach Abschluss des Experiments ausgeführt. Gleichzeitig wurde die initiale Konzentration dieser Stoffe in der beim Versuche angewandten Inkubationslösung vermittelst paralleler Versuche kontrolliert. Mindestens sechs solcher Kontroll-Analysen wurden bei jedem Experiment ausgeführt. Eine Ausnahme bildeten die Versuche, in denen die Retention von Calcium und Phosphor in den Grundlösungen studiert wurde und bei denen dieselben ein für allemal kein Calcium und Phosphor zu enthalten sich erwiesen.

a) *Die Bestimmung von Calcium* wurde gemäss der wohlbekannten titrimetrischen Methode von KRAMER und TISDALL (1921) ausgeführt, welche nach gewissen Abänderungen sich als die brauchbarste erwiesen hatte. Bei dem Ausfällen von Calciumoxalat erwies es sich als notwendig die Fällungsdauer bis auf 24 Stunden aus-

zudehnen, da bei kürzerer Fällungzseit die Bestimmungen keine sicheren Werte ergaben. Um die Empfindlichkeit der Methodik zu erhöhen, wurde beim Titrieren des ausgefällten Oxalats eine 1/200—1/250 n Kaliumpermanganatlösung anstatt der vorgeschriebenen 1/100 n Lösung angewandt. Nach langen Übungen gelang es eine für den Zweck der Versuche befriedigende Empfindlichkeit dieser Bestimmungsmethode zu erreichen. Somit wurde in Kontrollserien mit 10—11 mg %-igen Calciumstandardlösungen gewöhnlichein 1 % untersteigender Durchschnittsfehler erhalten.

b) *Die Bestimmung des Phosphors* geschah photometrisch nach der üblichen, von FISKE und SUBBAROW (1925) beschriebenen und von LOHMANN und JENDRASSIK (1926) modifizierten Methode. Vor der Bestimmung wurden Spuren von Eiweiss in der Inkubationslösung durch einen Zusatz von 4 ccm 10 %-iger Trichloressigsäure pro 1 ccm Lösung entfernt. Bei der Bestimmung des initialen Phosphorgehalts der Inkbationslösung wurde, obgleich kein Eiweiss vorhanden war, dasselbe Verfahren der Gleichförmigkeit wegen angewandt.

Der anorganische Phosphor wurde gemäss der obenerwähnten Methode unmittelbar aus dem Trichloressigsäurefiltrat bestimmt.

Die Bestimmung des organisch gebundenen Phosphors im Na-Glycerophosphat geschah nach derselben Methode aus der gesamten säurelöslichen Phosphorfraktion. Dieser wurde nach dreistündiger Verbrennung mit Schwefel- und Salpetersäure und nach nachträglicher Neutralisation mit Ammoniak, wobei p-Nitrophenol als Indikator angewandt wurde, aus dem Trichloressigsäurefiltrat berechnet.

Bei direkter Bestimmung von Phosphor aus Na-Glycerophosphat enthaltenden Lösungen konnte kein Phosphor nachgewiesen werden. Na-Glycerophosphat ist, wie bekannt, eine äusserst stabile Verbindung, aus welcher Phosphor sich schwerlich hydrolysieren lässt. Zur Beleuchtung dieses Sachverhalts wurde in einer Kontroll-Serie eine Na-Glycerophosphat enthaltende Lösung (10 mg % P) mit 2n Salzsäure in geschlossenen Probierröhren im Wasserbade bei 100° behandelt. Keine Abspaltung von Phosphor kam jedoch nach einer Hydrolysierungsdauer von bis auf 6 Stunden vor. Vollständige Hydrolyse fand erst nach dem obenerwähnten Verbrennungsverfahren statt.

Das Blut der Meerschweinchen wurde im Zusammenhange mit einigen der Versuche aufgehoben und Calcium aus dem Serum, sowie die anorganischen und organischen Phosphorfraktionen aus dem Vollblute nach dem obenerwähnten Verfahren bestimmt.

Die folgenden Verkürzungen wurden in allen Versuchen der Bestimmungen von Phosphor anegewandt:

anorg. P bedeutet den vermittelst der Fiske-Subbarowschen Methode direkt bestimmbaren anorganischen Phosphor. Somit ist der in $Na_2HPO_4 . 2H_2O$ enthaltene Phosphor in dieser Fraktion einbegriffen.

org. P bedeutet den organisch gebundenen Phsphor in Na-Glycerophosphat.

TCE—P ist der gesamte, aus den Trichloressigsäurefiltrat nach Verbrennung bestimmte, säurelösliche Phosphor. Der in Na-Glycerophosphat gebundene Phosphor ist also in TCE—P bei Versuchen, in denen Na-Glycerophosphat als Substrat zur Anwendung kam, enthalten.

IV. Versuche und Ergebnisse.

A. Aufnahme und Retention von Calcium und Phosphor in normalen Knochen.

Im Folgenden wird über orientierende Versuche, die die Aufnahme und Retention von Calcium und Phosphor in Knochen normaler Ratten und Meerschweinchen beleuchten, Bericht erstattet. Da wahrscheinlich eine Wechselwirkung im Calcium- und Phosphorstoffwechsel der Knochensubstanz besteht, habe ich es für nötig erachtet, die Aufnahme von Calcium und von Phosphor anfänglich getrennt zu untersuchen. Bei diesen Versuchen wurde also den basalen Inkubationslösungen entweder nur Calcium oder ausschliesslich Phosphor zugeführt, um die Affinität des Knochens zu den genannten Ionen möglichst genau studieren zu können.

Anfänglich hatte ich die Absicht, eine aufgenommene, bzw. abgegebene Calcium- und Phosphormenge per Gewichtseinheit der Präparatsubstanz zu berechnen, um einen einheitlichen Ausdruck für den in den Präparaten vorsichgegangenen Calcium- und Phosphorstoffwechsel zu finden. Es erwies sich jedoch, dass diese Calcium- und Phosphorwerte, obgleich sie im allgemeinen am höchsten in den grösseren Präparaten waren, in keinem exakten mathematischen Verhältnis zum Gewicht der Präparate standen, weshalb es mir als angeraten erschien, die Calcium- und Phosphorwerte, sowie die Präparatengewichte in absoluten Massen aufzugeben. In Anbetracht jedoch des verhältnismässig grossen Unterschiedes im Gewicht der Vorder- und der Hinterbeinspräparate wurde der Calcium- und Phosphorstoffwechsel in denselben getrennt berechnet. Ein solches Verfahren erschien desto mehr berechtigt

zu sein, da HAMMETT (1928) hat Verschiedenheiten in dem Aschen-Gehalt der vorderen und hinteren Extremitätsknochen von Ratten beobachten können.

1. *Aufnahme von Calcium.*

In den folgenden Versuchserien ist die Adsorption von Calcium in 16 Knochenpräparaten von insgesamt 9 normalen Ratten beobachtet worden, wobei 3 basale Inkubationslösungen, nämlich destilliertes Wasser, 0.85 %-ige Natriumkloridlösung, sowie die Grundlösung ROBISONS zur Anwendung gekommen sind. Der Calciumgehalt in diesen Lösungen betrug 10—11 mg % (absolute Menge 0,1—0.11 mg Calcium). Die Menge der Inkubationslösungen war je 10 ccm.

TABELLE 1. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm destilliertes Wasser)

Nr.	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 1	HRU	240	10.0	9.5	0.05
	HRU	240	10.0	9.3	0.07
R 2	HRU	280	10.0	9.8	0.02
	FT	660	10.0	9.2	0.08
R 3	HRU	490	10.0	9.2	0.08
	FT	1355	10.0	9.1	0.09
Durchschnittswert:					
Vorderbeine		313	10.0	9.5	0.05
Hinterbeine		1008	10.0	9.2	0.08

Aus den Tabellen 1—3 ist zu ersehen, dass eine Aufnahme von Calcium in die Knochenpräparate bei allen Versuchen stattgefunden hat, obgleich die analytischen Unterschiede verhältnismässig klein sind. Bei den Versuchen erwies es sich, dass die grösseren Hinterbeine, absolut gemessen, mehr Calcium als die kleineren Vorderbeine aufnahmen. Was die aufgenommene Calciummenge selbst betrifft, so scheint dieselbe von der Zusammensetzung der

TABELLE 2. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)

Nr.	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 4	HRU	600	10.8	10.5	0.03
	FT	1030	10.8	10.1	0.07
R 5	HRU	720	10.8	10.6	0.02
	FT	1100	10.8	9.8	0.10
R 6	HRU	890	10.8	10.3	0.05
	FT	950	10.8	9.6	0.12
Durchschnittswert:					
Vorderbeine		**737**	**10.8**	**10.5**	**0.03**
Hinterbeine		**1027**	**10.8**	**9.8**	**0.10**

TABELLE 3. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm Robisons Grundlösung)

Nr.	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 7	HRU	720	11.0	10.2	0.08
R 8	FT	900	11.0	9.6	0.14
R 9	HRU	850	11.0	10.5	0.05
	T	850	11.0	10.7	0.03
Durchschnittswert:					
Vorderbeine		**785**	**11.0**	**10.4**	**0.06**
Hinterbeine		**875**	**11.0**	**10.2**	**0.08**

basalen Inkubationslösung in keinem nennenswerten Ausmasse abzuhängen.

2. *Retention von Calcium.*

Zur Beleuchtung dieses Problems wurden in den nachfolgenden Experimenten Knochenpräparate normaler Ratten in 10 ccm 0.85 %-iger Natriumchloridlösung ohne Zusatz von Calcium inkubiert. Bei diesem Versuche wurden 11 Knochenpräparate von 9 Ratten angewandt.

TABELLE 4. *Retention von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)

Nr.	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Abgegebenes Ca mg
R 10	HRU	555	0	4.7	0.47
	HRU	645	0	3.2	0.32
	FT	1090	0	5.6	0.56
	FT	1195	0	4.4	0.44
R 11	HRU	550	0	4.0	0.40
	HRU	620	0	4.2	0.42
	FT	1300	0	5.4	0.54
R 12	HRU	570	0	5.2	0.52
	HRU	540	0	4.9	0.49
	FT	1450	0	5.1	0.51
	FT	1300	0	5.8	0.58
Durchschnittswert:					
Vorderbeine		**580**	**0**	**4.4**	**0.44**
Hinterbeine		**1267**	**0**	**5.3**	**0.53**

Die Tabelle 4 zeigt deutlich, dass eine Auswanderung von *Calcium aus normalen Knochen in die dieselben umgebende physiologische Natriumchloridlösung* stattgefunden hat. Die aus den Vorderbeinen abgegebene Calciummenge scheint im Verhältnis zum Gewicht des Präparats am grössten zu sein.

3. *Aufnahme von anorganischem Phosphor.*

Bei der Untersuchung der Fähigkeit normaler Knochen anorganisches Phosphat aufzunehmen sind in den folgenden Experimenten 30 Knochenpräparate von 4 normalen Meerschweinchen zur Anwendung gekommen. Die Menge der Inkubationslösung war je 10 ccm.

Aus der Tabelle 5 geht deutlich hervor, dass Knochen normaler Meerschweinchen die Fähigkeit anorganischen Phosphor aus 20—50 mg %-igen Phosphorlösungen aufzunehmen nicht zu besitzen scheinen. Mit der Ausnahme von 3 Fällen, in denen der finale anor-

TABELLE 5. *Aufnahme von anorganischem Phosphor in Meerschweinchenknochen.* (10 ccm Robisons Grundlösung)

Nr.	Knochen	Gewicht des Präparats mg	Initialer anorg. P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 1	H	1070	20.0	20.0	20.0	0
	H	1100	20.0	21.1	21.1	—0.11
	RU	770	20.0	20.0	20.0	0
	RU	770	20.0	22.3	22.3	—0.23
	F	1800	20.0	22.3	22.3	—0.23
	F	1780	20.0	22.9	22.9	—0.29
	T	1260	20.0	21.1	21.1	—0.11
	T	1260	20.0	21.7	21.7	—0.17
M 2	H	900	25.0	30.4	24.3	—0.54
	H	920	25.0	32.1	25.7	—0.71
	RU	630	25.0	29.6	25.7	—0.46
	RU	630	25.0	28.8	27.1	—0.38
	F	1520	25.0	30.4	27.1	—0.54
	F	1480	25.0	29.6	26.5	—0.46
	T	1070	25.0	30.4	25.7	—0.54
	T	1050	25.0	28.8	27.1	—0.38
M 3	H	880	25.0	26.8	24.3	—0.18
	H	880	25.0	28.3	22.3	—0.33
	RU	590	25.0	28.3	25.0	—0.33
	F	1500	25.0	26.1	25.7	—0.11
	T	940	25.0	26.8	26.4	—0.18
	T	940	25.0	27.5	26.4	—0.25
M 4	H	590	50.0	51.5	51.5	—0.15
	H	590	50.0	54.5	53.0	—0.45
	RU	450	50.0	54.5	51.5	—0.45
	RU	450	50.0	56.0	54.5	—0.60
	F	1040	50.0	53.5	53.5	—0.35
	F	1040	50.0	53.0	50.0	—0.30
	T	750	50.0	53.0	53.0	—0.30
	T	770	50.0	56.0	56.0	—0.60

ganische Phosphorgehalt gegenüber dem initialen ein wenig verringert war, zeigt die überwiegende Mehrheit der Versuche Werte auf, welche darauf deuten, dass, umgesetzt, anorganischer Phosphor in die Inkubationslösung ausgewandert war. Ausser dem ausgeschiedenen anorganischen Phosphor waren in einigen Fällen kleine Mengen organisch gebundenen Phosphors in die umgebende Lösung während der Inkubationszeit ebenso ausgewandert. Somit hat kein Versuch aufzuweisen, dass der gesamte säurelösliche Phosphor während der Inkubation in der umgebenden Lösung eine Verminderung erfahren hätte.

Die Beobachtung, dass Knochen normaler Meerschweinchen die Fähigkeit anorganischen Phosphor aufzunehmen nicht zu besitzen scheinen, bestätigt eine frühere Wahrnehmung, in der dieselbe Erscheinung bei entsprechenden Inkubationsversuchen mit Ratten-Knochen festgestellt werden konnte (v. KRAMER, LANDTMAN, SIMOLA 1940, 1941). Bei diesen Versuchen wurde eine Phosphorkonzentration von bis auf 100 mg % angewandt.

4. *Aufnahme von organischem Phosphor.*

Die Beobachtung, dass Knochen normaler Meerschweinchen keine Aufnahmefähigkeit für anorganischen Phosphor zu besitzen scheinen, gab Anlass zu Versuchen, in denen organisch gebundener Phosphor als Substrat in den Inkubationslösungen angewandt wurde. In den in der Tabelle 6 angeführten Versuchen wurde die Aufnahme von Na-Glycerophosphat in Knochen von normalen Meerschweinchen aus der Grundlösung ROBISONS studiert. Diese orientierende Untersuchungen umfassten insgesamt 12 Knochen zweier Meerschweinchen.

Ein Überblick der in Tabelle 6 angeführten Ergebnisse zeigt, dass in allen Versuchen eine deutlich nachweisbare Aufnahme von organisch gebundenem Phosphor statt gefunden hat.

Nach der Inkubation konnte in der Versuchslösung, welche anfänglich keinen Phosphor in einer direkt bestimmbaren Form enthielt, bei den verschiedenen Versuchen anorganischer Phosphor in Mengen von etwa 8—9 mg % nachgewiesen werden. Der Ursprung dieser direkt bestimmbaren Phosphorfraktion dürfte teilweise darin zu suchen sein, dass ein Teil des organisch gebundenen

TABELLE 6. *Aufnahme von organischem Phosphor in Meerschweinchenknochen.* (10 ccm Robisons Grundlösung)

Nr.	Knochen	Gewicht des Präparats mg	Initialer org. P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 5	H	1100	18.0	13.0	8.0	0.50
	H	1130	18.0	14.3	8.0	0.37
	RU	700	18.0	14.7	9.0	0.33
	RU	750	18.0	15.1	9.4	0.29
	F	1700	18.0	16.0	9.2	0.20
	F	1700	18.0	13.4	8.7	0.46
	T	1150	18.0	13.8	8.2	0.42
	T	1170	18.0	16.5	9.2	0.15
M 6	H	760	18.4	14.2	6.8	0.42
	RU	520	18.4	15.2	7.8	0.32
	F	1260	18.4	14.7	8.5	0.37
	T	850	18.4	14.2	8.0	0.42
Durchschnittswert:						
Vorderbeine		827	18.1	14.4	8.2	0.37
Hinterbeine		1305	18.1	14.8	8.6	0.33

Phosphors infolge des hydrolysierenden Einflusses der Knochen-Phosphatasen während der Inkubation in der Versuchslösung zersetzt wird. Auch kann die Möglichkeit in Erwägung kommen, dass wenigstens ein Teil dieses anorganischen Phosphors aus den Knochen in die Inkubationslösung ausgewandert sein dürfte. Im Folgenden wird diese letztere Möglichkeit näher untersucht werden.

5. *Retention von anorganischem Phosphor.*

In Übereinstimmung mit den Versuchen, in denen die Retention von Calcium in Knochenpräparaten studiert wurde, ist in den entsprechenden Experimenten über die Retention von anorganischem Phosphor in Knochen eine 0.85 %-ige Natriumchloridlösung zur Inkubation angewandt worden. Dreien Ratten entnommene 12 Knochenpräparate sind hier zur Anwendung gekommen. Ebenso wie in den bisher beschriebenen Experimenten betrug die Menge der Inkubationslösung je 10 ccm.

TABELLE 7. *Retention von anorganischem Phosphor in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)

Nr.	Knochen	Gewicht des Präparats mg	Initialer anorg. P mg %	Finaler anorg. P mg %	Abgegebener anorg. P mg
R 10	HRU	555	0	2.4	0.24
	HRU	645	0	2.0	0.20
	FT	1090	0	3.4	0.34
	FT	1195	0	3.8	0.38
R 12	HRU	570	0	3.8	0.38
	HRU	540	0	3.3	0.33
	FT	1450	0	4.8	0.48
	FT	1300	0	4.1	0.41
R 13	HRU	650	0	3.3	0.33
	HRU	650	0	2.6	0.26
	FT	1485	0	6.0	0.60
	FT	1505	0	6.8	0.68
Durchschnittswert:					
Vorderbeine		602	0	2.9	0.29
Hinterbeine		1338	0	4.8	0.48

Die Tabelle 7 zeigt in den gesamten Fallen eine deutlich nachweisbare Auswanderung von anorganischem Phosphor aus den Knochenpräparaten in die Inkubationslösung auf. Der Phosphor scheint somit, ähnlich wie es mit Calcium der Fall ist, in der Knochensubstanz nicht stabil fixiert zu sein, da ein Teil desselben in das umgebende Medium frei auswandern kann. Die abgegebene Phosphormenge scheint in den Versuchen mit dem Gewichte der Knochenpräparate einigermassen proportional zu sein.

B. Calcium- und Phosphorstoffwechsel im Knorpelgewebe.

In den folgenden Versuchen wurde die Aufmerksamkeit auf das Studium des Metabolismus von Calcium und Phosphor im Knorpelgewebe gegenüber diesem Stoffwechsel in der Knochensubstanz gerichtet. Zu diesem Zwecke wurden einem Kalbembryo die langen Knochen der Extremitäten entnommen, welche genügend

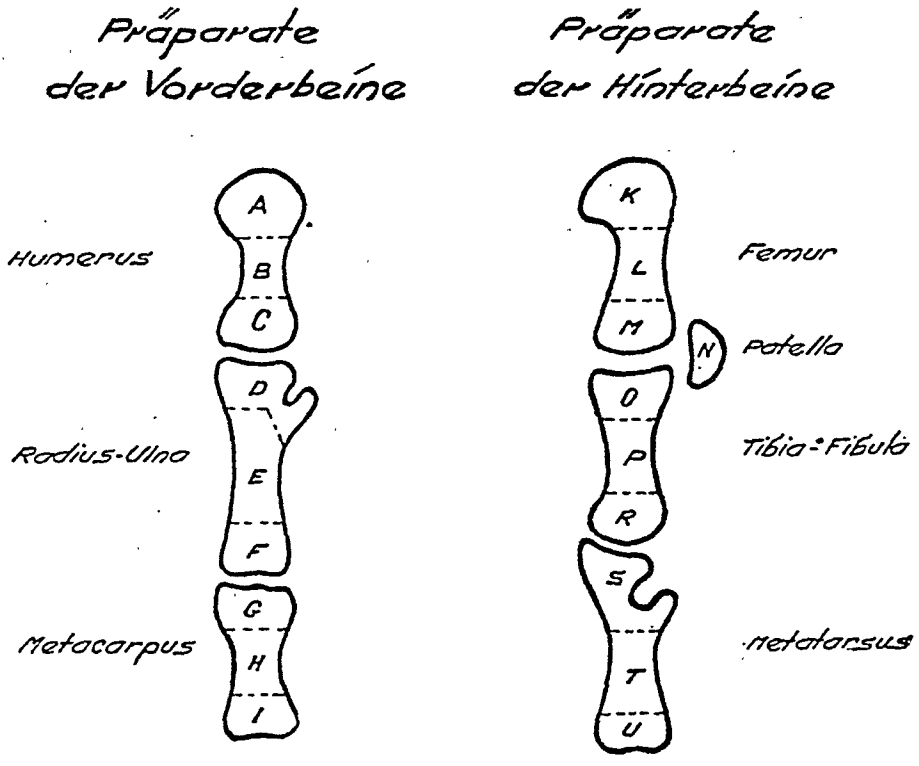

Abbildung 1.

gross waren um die Inkubation der Knochen- und Knorpelteile getrennt zu ermöglichen. Die Epiphysenknorpeln waren schwach durchsichtig im Gegensatz zu den Diaphysen, welche das Aussehen von fertig ausgebildetem Knochen hatten. Die Isolierung der Epiphysen wurde durch einen Schnitt mit einer Rasierklinge der Grenze zwischen dem Knorpel und Knochen entlang bewerkstelligt. Die Schnitte hinterliessen keine blutenden Flächen. In der obigen schematischen Zeichnung stellen die Strichlinien die zwecks Abscheidung der verschiedenen Präparate gemachten Schnitte dar.

Insgesamt kamen 27 Präparate zur Anwendung (12 Knochen- und 15 Knorpelpräparate). Die Numerierung und Verteilung der Präparate gehen aus der Tabelle 8 hervor.

Beim Studium des Calcium- und Phosphormetabolismus in den Epiphysen- und Diaphysenpräparaten war, ebenso wie in den

TABELLE 8. *Numerierung und Verteilung der Präparate.*

Präparate der							
Vorderbeine				Hinterbeine			
dx		sin		dx		sin	
Nr.	Präparat	Nr.	Präparat	Nr.	Präparat	Nr.	Präparat.
1	A	8	A+C	14	K+M	21	K+M
2	B	9	B	15	L	22	L
3	C	10	D+F	16	N	23	N
4	D + F	11	E	17	O+R	24	O+R
5	E	12	G+I	18	P	25	P
6	G + I	13	H	19	S+U	26	S+U
7	H			20	T	27	T

früheren Versuchen, die Aufmerksamkeit sowohl auf die Aufnahme wie auch auf die Retention dieser Stoffe gerichtet. Die Inkubation geschah in 50 ccm fassenden Probierrohren, enthaltend 25 ccm Lösung.

1. *Aufnahme von Calcium und Phosphor.*

Die Aufnahme von Calcium und Phosphor wurde in den den rechten Extremitäten entnommenen Präparaten studiert. Als Grundlösung wurde bei diesen Versuchen die Robisonsche Basallösung angewendt, welcher Calcium, sowie organischer und anorganischer Phosphor zugeführt wurden. Bei der Herstellung der Inkubationslösung entstand eine schwach bemerkbare anfängliche Trübung, welche vor den Versuchen vermittelst Filtrieren beseitigt wurde. Die klare, filtrierte Inkubationslösung enthielt Calcium und Phospor in folgenden Mengen:

$CaCl_2 \cdot 6\,H_2O$	12.0 mg	%	Ca	(absolute	Menge	3.00 mg	Ca)
$Na_2HPO_4 \cdot 2\,H_2O$	4.4 »	»	P	(»	»	1.10 »	P)
Na-Glycerophosphat	2.9 »	»	P	(»	»	0.73 »	P)
[TCE-P	7.3 »	»	P	(»	»	1.83 »	P)]

Aus den Tabellen 9 und 10 geht hervor, dass die Knorpelpräparate bedeutend mehr Calcium und Phosphor als die Knochen-

TABELLE 9. *Aufnahme von Calcium und Phosphor in Knochendiaphysen.*
(25 ccm Robisons Grundlösung)

Nr.	Gewicht des Präparats mg	Finales Ca mg %	Aufgenommenes Ca mg	Finale TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
2	3100	11.7	0.08	6.8	6.8	0.13
5	5500	10.7	0.33	6.2	5.6	0.28
7	3400	7.1	1.23	7.2	7.2	0.03
15	5000	9.3	0.68	6.3	6.3	0.25
16	5200	11.8	0.05	5.9	5.9	0.35
20	2900	9.5	0.63	6.3	6.3	0.25
Durchschnittswert:	**4183**	**10.0**	**0.50**	**6.5**	**6.4**	**0.20**

TABELLE 10. *Aufnahme von Calcium und Phosphor in Epihysenknorpeln.*
(25 ccm Robisons Grundlösung)

Nr.	Gewicht des Präparats mg	Finales Ca mg %	Aufgenommenes Ca mg	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
1	4200	8.2	0.95	4.0	4.0	0.83
3	5400	9.0	0.75	5.2	5.2	0.53
4	5350	5.9	1.53	4.9	4.9	0.60
6	4900	7.0	1.25	4.3	4.3	0.75
14	7200	8.1	1.00	5.9	5.9	0.35
16	2700	5.9	1.53	4.6	4.6	0.68
17	5800	8.8	0.80	5.3	5.3	0.50
19	4000	11.0	0.25	5.7	5.7	0.40
Durchschnittswert:	**4944**	**8.0**	**1.01**	**5.0**	**5.0**	**0.58**

Präparate aufgenommen hatten. Die in die Diaphysen aufgenommene Calciummenge war im Durchschnitt 0.50 mg und die in die Epiphysen 1.01 mg. Die entsprechenden Werte für aufgenommenen Phosphor waren 0.20 mg, bzw. 0.58 mg. Das durchschnittliche Gewicht der Knorpelpräparate war etwas höher (4944 mg) als das der Knochenpräparate (4183 mg), jedoch ist in Anbetracht der

einzelnen Versuchsergebnisse diese Differenz so klein, dass sie allein den Unterschied zwischen der Aufnahme von Calcium und Phosphor kaum motivieren könnte. Bei allen Versuchen mit Ausnahme der Nr. 5 fand sich nach der Inkubation die ganze Phosphormenge in anorganischer, direkt bestimmbarer Form in der Versuchslösung vor. Nur in den Versuchen 1 und 6 unterstieg der finale gesamte Phosphorspiegel die initiale anorganische Phosphormenge der Inkubationslösung (4.4 mg % P), was vielleicht damit erklärt

TABELLE 11. *Retention von Calcium und Phosphor in Knochendiaphysen.* (25 ccm Robisons Grundlösung)

Nr.	Gewicht des Präparats mg	Finales Ca mg %	Abgegebenes Ca mg	Finaler TCE-P mg %	Finaler anorg. P mg %	Abgegebener P mg
9	5300	8.4	2.10	3.3	3.3	0.83
11	5600	6.0	1.50	5.5	4.0	1.38
13	3300	5.5	1.38	4.3	4.3	1.08
22	5100	7.4	1.85	4.6	4.6	1.15
25	5500	8.0	2.00	2.7	2.7	0.68
27	3100	4.3	1.08	3.5	3.2	0.88
Durchschnittswert:	4650	6.6	1.65	4.0	3.7	1.00

TABELLE 12. *Retention von Calcium und Phosphor in Epiphysenknorpeln.* (25 ccm Robisons Grundlösung)

Nr.	Gewicht des Präparats mg	Finales Ca mg %	Abgegebenes Ca mg	Finaler TCE-P mg %	Finaler anorg. P mg %	Abgegebener P mg
8	7000	5.7	1.43	1.5	1.5	0.38
10	5900	5.1	1.28	1.8	1.8	0.45
12	3400	4.0	1.00	3.4	3.4	0.85
21	6700	4.9	1.23	2.6	2.6	0.65
23	2800	3.9	0.98	2.2	2.2	0.55
24	4100	4.8	1.20	3.2	3.2	0.80
26	3300	3.9	0.98	2.4	2.4	0.60
Durchschnittswert:	4743	4.6	1.15	2.4	2.4	0.60

werden könnte, dass die Präparate vorzugsweise Phosphor in organischer Form aufgenommen hatten. Andererseits dürfte auch die Möglichkeit nicht ausgeschlossen werden können, dass anorganischer Phosphor vom Glycerophosphat infolge der Phosphatasewirksamkeit in den Präparaten abgespaltet worden ist.

2. *Retention von Calcium und Phosphor.*

Die Retention von Calcium und Phosphor wurde in den den linken Extremitäten entnommenen Knorpel- und Knochen-Präparate studiert. Zur Inkubation wurde die Grundlösung Robisons ohne Zusatz von Calcium oder Phosphor angewandt. Die Ergebnisse sind aus den Tabellen 11 und 12 ersichtlich.

Ein Vergleich der Ergebnisse dieser beiden Versuchsserien zeigt, dass die Knorpelpräparaten Calcium und Phosphor besser als die Diaphysen fixiert haben. Obgleich das Durchschnittsgewicht der Epiphysenpräparate (4743 mg) etwas höher als das der Diaphysen (4650 mg) war, gaben die ersteren an die Inkubations-Lösung durchschnittlich weniger Calcium und Phosphor (1.15 mg Ca, 0.60 mg P) als die letzteren (1.65 mg Ca, 1.00 mg P) ab. Der ausgewanderte Phosphor lag, ausser in den Versuchen mit Knochen-Präparaten NNr. 11 und 27, nur in anorganischer, direkt bestimmbaren Form vor. Abgesehen von einer rein physikalischen Auswanderung von nur anorganischem Phosphorverbindungen aus den Präparaten besteht auch die Möglichkeit, dass die Überführung dieses Phosphors in die Lösung in irgendeinem Verhältnis zur Phosphataseaktivität in den Präparaten steht.

C. Der Einfluss von gewissen Zellengiften auf die Aufnahme und Retention von Calcium und Phosphor im Knochengewebe.

Um im Ramen dieser in vitro ausgeführten Versuche dem Mechanismus des Calcium- und Phosphorstoffwechsels, wenn möglich, besser auf die Spur zu kommen, wurde im Folgenden die Aufmerksamkeit auf das Studium der Adsorption und Retention von Calcium und Phosphor in den Präparaten in der Anwesenheit

gewisser Zellengifte in der Inkubationslösung gerichtet. Als solche Stoffe wurden Formaldehyd und in einem der Versuche Toluol angewandt, welche, wie bekannt, eine toxische Wirkung auf verschiedene biochemische Prozesse ausüben.

Die Methodik der Versuche war wie folgt: Einen und denselben Tieren — 8 Meerschweinchen kamen bei diesen Versuchen zur Anwendung — wurden die homologen Extremitätsknochen für zwei Versuchsserien entnommen. Die gesamte Anzahl der Präparate war 64. Die Aufnahme und Retention von Calcium und Phosphor geschah in den beiden Serien unter im Übrigen gleichen Bedingungen, mit dem Unterschiede aber, dass in einer der Serien der Inkubationslösung Zellengift zugeführt wurde. Der Lösung der anderen Serie wurde eine entsprechende Volummenge physiologischer Natriumchloridlösung hinzugefügt.

a. *Die Wirkung von Formaldehyd auf die Aufnahme von Calcium und organischem Phosphor.* Die Tabelle 13 zeigt die Aufnahme von Calcium und Phosphor in insgesamt 24 Knochenpräparaten von 3 Meerschweinchen. Die homologen Knochen der verschiedenen Tiere wurden hierbei auf zwei Serien verteilt, in einer von denen die Inkubationslösung 3.5 % Formaldehyd enthielt. Als Basal-Lösung wurde die Grundlösung Robisons angewandt, welche ausserdem Calciumchlorid und Na-Glycerophosphat als Substrate enthielt. Die initiale Calciummenge variierte zwischen 12.5—14.0 mg %, wogegen der Phosphorgehalt 16 mg % war. Die Ergebnisse weisen einen auffallenden Unterschied in den beiden Serien auf. Indem alle Präparate der Kontrollversuche Calcium aufgenommen hatten — das adsorbierte Calcium betrug im Durchschnitt in den Vorder- und Hinterbeinsknochen 0.13 bzw. 0.16 mg — ist in der Formaldehydserie der finale Calciumgehalt der Inkubationslösung im Durchschnitt etwas höher als der initiale, welches zeigt, dass keine Aufnahme, aber eher eine Abgabe von Calcium aus den Knochen statt gefunden hat. Die aus den Knochen der Vorderbeine abgeschiedene Calciummenge betrug durchschnittlich 0.05 mg und die aus denen der Hinterbeine 0.09 mg. Diese Werte sind zwar so klein, dass man von einer direkten Auswanderung von Calcium aus den Knochen wohl kaum reden kann, jedoch erbringen sie einen deutlichen Beweis dafür, dass das Formaldehyd den Calciumadsorptionsmechanismus gelähmt hat. Von irgend-

TABELLE 13. *Der Einfluss von Formaldehyd (3,5 %) auf die Aufnahme von Calcium und organischem Phosphor in Meerschweinchenknochen.*
(10 ccm Robisons Grundlösung)

Nr.	Knochen	Gewicht des Präparats mg	Kontrolle							Gewicht des Präparats mg	3.5 % Formaldehyd						
			Ca			P					Ca			P			
			Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg %	Initialer org. P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg		Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg %	Initiales org. P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 7	H	970	12.5	11.1	0.14	16.0	11.5	1.6	0.45	1000	12.5	12.4	0.01	16.0	18.6	0.7	—0.26
	RU	570	12.5	10.8	0.17	16.0	13.0	3.2	0.30	570	12.5	12.6	—0.01	16.0	16.0	0.0	0.00
	F	1450	12.5	10.6	0.19	16.0	13.4	3.4	0.26	1450	12.5	12.0	0.05	16.0	18.0	0.0	—0.20
	T	1060	12.5	10.9	0.16	16.0	13.0	4.0	0.30	1060	12.5	12.5	0.00	16.0	17.0	0.0	—0.10
M 8	H	850	14.0	13.8	0.02	16.0	12.8	4.0	0.32	850	14.0	15.5	—0.15	16.0	16.2	1.3	—0.02
	RU	550	14.0	12.0	0.20	16.0	13.1	4.1	0.29	560	14.0	15.0	—0.10	16.0	16.7	2.2	—0.07
	F	1275	14.0	12.3	0.17	16.0	11.8	4.8	0.42	1270	14.0	16.6	—0.26	16.0	15.4	2.9	0.06
	T	820	14.0	12.0	0.20	16.0	13.5	3.8	0.25	850	14.0	16.0	—0.20	16.0	15.8	2.0	0.02
M 9	H	910	14.0	13.4	0.06	16.0	12.2	5.5	0.38	880	14.0	14.5	—0.05	16.0	17.4	3.0	—0.14
	RU	570	14.0	12.3	0.17	16.0	11.8	4.9	0.42	560	14.0	13.8	0.02	16.0	17.2	2.2	—0.12
	F	1450	14.0	12.9	0.11	16.0	11.4	6.5	0.46	1400	14.0	14.8	—0.08	16.0	19.3	3.2	—0.33
	T	870	14.0	12.6	0.14	16.0	13.1	5.7	0.29	880	14.0	14.4	—0.04	16.0	17.4	3.0	—0.14
Durchschnittswert:																	
Vorderbeine		737	13.5	12.2	0.13	16.0	12.4	3.9	0.36	737	13.5	14.0	—0.05	16.0	17.0	1.6	- 0.10
Hinterbeine		1154	13.5	11.9	0.16	16.0	12.7	4.7	0.33	1152	13.5	14.4	0.09	16.0	17.2	1.9	0.12

welchen nennenswerten analytischen Fehlermöglichkeiten kann nicht die Rede sein, da es sich bei Kontrollversuchen erwiesen hatte, dass das Formaldehyd keinen Einfluss auf die Empfindlichkeit der Calciumbestimmungen ausübte.

Der Unterschied in der Aufnahme von organisch gebundenem Phosphor ist ebenso ausgesprochen. Die Kontrollserie zeigt, dass in sämtlichen Präparaten eine Aufnahme stattgefunden hat. Im Durchschnitt betrug hier der adsorbierte Phosphor 0.36 mg für die Vorderbein- und 0.33 mg für die Hinterbeinpräparate. Dagegen fand in der Anwesenheit von Formaldehyd in der überwiegenden Anzahl der Fälle eine Auswanderung von Phosphor aus den Knochen statt. Die abgegebene Phosphormenge war im Durchschnitt 0.10 mg für die Präparate der Vorder- und 0.12 mg für die der Hinterbeine. Auffallend ist ausserdem der Umstand, dass die finale anorganische Phosphorfraktion der Inkubationslösung bei den Kontrollversuchen durchschnittlich ungefähr doppelt so gross (3.9 mg %, 4.7 mg %) war wie in der Anwesenheit von Formaldehyd (1.6 mg %, 1.9 mg %). Die letztere Serie weist drei Fälle auf, in denen nach Abschluss des Experiments die Inkubationslösung kein Phosphor in einer direkt bestimmbaren Form aufwies. Bei Kontrollanalysen erwies es sich, dass Formaldehyd auf die Empfindlichkeit der Phosphorbestimmung keine Wirkung ausübte.

b. *Die Wirkung von Toluol auf die Aufnahme von organischem Phosphor.* In diesem Versuche sind insgesamt 24 Knochenpräparate von 3 Meerschweinchen zur Anwendung gekommen. Die Inkubationslösung — auch in diesem Falle die Grundlösung Robisons — enthielt als Substrat Na-Glycerophosphat (19 mg % P).

Die aus der Tabelle 14 ersichtlichen Ergebnisse zeigen, dass Toluol ebenso wie Formaldehyd auf die Aufnahme von organischem Phosphor hemmend wirkt. Eine vollständige Lähmung der Adsorption ist jedoch nicht aufgetreten, da die meisten Präparate Phosphor in mehr oder minder grossen Mengen aufgenommen hatten, jedoch waren die in den einzelnen Versuchen in der Anwesenheit von Toluol aufgenommenen Phosphormengen im Vergleich zu denen der Kontrollserie beinahe durchgehends kleiner. Die Mittelwerte weisen aufgenommene Phosphormengen von 0.44 mg und 0.33 mg in den Kontrollpräparaten auf, wogegen die entsprechenden Zahlen in der Toluolserie 0.11 und 0.05 mg sind. Die finale anorganische

TABELLE 14. *Der Einfluss von Toluol (2 %) auf die Aufnahme von organischem Phosphor in Meerschweinchenknochen.*
(10 ccm Robisons Grundlösung)

		Kontroll					2 % Toluol				
Nr.	Knochen	Gewicht des Präparats mg	Initialer org. P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg	Gewicht des Präparats mg	Initialer org. P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 10	H	750	19.0	15.1	11.0	0.39	770	19.0	17.4	8.5	0.16
	RU	500	19.0	16.4	10.0	0.26	450	19.0	15.8	9.1	0.32
	F	1180	19.0	19.8	11.6	—0.08	1190	19.0	24.4	10.3	—0.54
	T	790	19.0	16.3	12.7	0.27	830	19.0	16.8	11.0	0.22
M 11	H	970	19.0	15.0	6.8	0.40	950	19.0	20.4	6.8	—0.14
	RU	630	19.0	15.6	6.9	0.34	650	19.0	19.8	7.4	—0.08
	F	1450	19.0	17.2	6.8	0.18	1440	19.0	17.2	7.6	0.18
	T	1000	19.0	14.6	7.1	0.44	1050	19.0	18.1	7.1	0.09
M 12	H	1020	19.0	13.4	3.4	0.56	990	19.0	17.2	5.1	0.18
	RU	700	19.0	12.3	6.1	0.67	670	19.0	16.6	6.9	0.24
	F	1650	19.0	13.4	6.4	0.56	1670	19.0	17.6	4.6	0.14
	T	1150	19.0	12.7	3.6	0.63	1150	19.0	16.6	5.1	0.24
Durchschnittswert:											
Vorderbeine		**762**	**19.0**	**14.6**	**7.4**	**0.44**	**747**	**19.0**	**17.9**	**7.3**	**0.11**
Hinterbeine		**1203**	**19.0**	**15.7**	**8.0**	**0.33**	**1222**	**19.0**	**18.5**	**7.6**	**0.05**

TABELLE 15. *Der Einfluss von Formaldehyd (3.5 %) auf die Retention von Calcium und Phosphor in Meerschweinchenknochen.* (10 ccm Robisons Grundlösung)

Kontroll							3.5 % Formaldehyd				
Nr.	Knochen	Gewicht des Präparats mg	Initiale Ca und P mg %	Abgegebenes Ca mg	Abgegebener P		Gewicht des Präparats mg	Initiale Ca und P mg %	Abgegebenes Ca mg	Abgegebener P	
					TCE-P mg	anorg. P mg				TCE-P mg	anorg. P mg
M 13	H	880	0	0.16	0.29	0.29	860	0	0.21	0.32	0.23
	RU	650	0	0.19	0.24	0.23	610	0	0.21	0.32	0.24
	F	1400	0	0.20	0.32	0.32	1360	0	0.28	0.72	0.21
	T	900	0	0.17	0.50	0.23	930	0	0.27	0.81	0.21
M 14	H	790	0	0.20	0.35	0.29	790	0	0.24	0.53	0.24
	RU	550	0	0.14	0.29	0.27	545	0	0.20	0.47	0.15
	F	1290	0	0.26	0.34	0.32	1260	0	0.28	0.62	0.29
	T	830	0	0.21	0.24	0.24	820	0	0.26	0.47	0.20
Durchschnittswert:											
Vorderbeine		**718**	**0**	**0.17**	**0.29**	**0.27**	**701**	**0**	**0.22**	**0.41**	**0.22**
Hinterbeine		**1105**	**0**	**0.21**	**0.35**	**0.28**	**1093**	**0**	**0.27**	**0.66**	**0.23**

Phosphorfraktion wies bedeutende Schwankungen auf, aber war durchschnittlich etwas grösser als in dem vorherigen Experiment, jedoch hatten auch in diesem Falle die Kontrollversuche im Durchschnitt etwas höhere Werte als in der »Vergiftungsserie» aufzuweisen (7.4 und 8.0 mg % gegen 7.3 und 7.6 mg %). Gleich dem Formaldehyd erwies sich auch das Toluol auf die Empfindlichkeit der Phosphorbestimmungen keine störende Wirkung auszuüben.

c *Die Wirkung von Formaldehyd auf die Retention von Calcium und Phosphor.* Die Tabelle 15 zeigt die Wirkung von Formaldehyd auf die Retention von Calcium und Phosphor in normalen Meerschweinchenknochen. Insgesamt wurden zweien Tierchen entnommene 16 Präparate zu diesen Versuchen angewandt.

Ein Vergleich der Ergebnisse dieser beiden Serien zeigt, dass die Knochen in Anwesenheit von Formaldehyd an die Inkubations-Lösung konsequent etwas mehr Calcium als die homologen Präparate der Kontrollversuche abgaben (im Durchschnitt 0.17 und 0.21 mg entgegen bzw. 0.22 und 0.27 mg). Dieser Unterschied ist mehr auffallend, was die totale abgegebene säurelösliche Phosphorfraktion betrifft (durchschnittlich 0.29 mg und 0.35 mg in der Kontrollserie, aber 0.41 mg und 0.66 mg in der Anwesenheit von Formaldehyd). Auffallend ist ausserdem der Umstand, dass der bei den Kontrollversuchen ausgeschiedene Phosphor zum grössten Teil in anorganischer Form vorlag, wogegen derselbe in der Formaldehydserie nur annäherend die Hälfte der Phosphorfraktion ausmachte, was vielleicht teilweise als ein Ausdruck einer gestörten Phosphatasetätigkeit in den Knochen aufgefasst werden kann.

D. Bakterienkontrolle.

Obgleich die Versuche so sauber wie möglich ausgeführt wurden konnten sie dennoch nicht unter vollkommen sterilen Verhältnissen ausgeführt werden. Bei dem Präparieren des Versuchs-Materials wurden den Präparaten unfreiwillig durch Berührung der Hände des Operateurs sowie aus der umgebenden Luft Bakterien zugeführt. Um annähernd beurteilen zu können, um welche Bakterienmengen es sich bei den Versuchen gehandelt haben

TABELLE 16. *Bakterienzüchtung aus Versuchslösungen nach abgeschlossener Inkubation.*

Versuchsmaterial		Anzahl der sämtlichen Kolonien		Anzahl der Bakterien pro ccm der Inkubationslösung
Tabelle	*Präparat*	24 Stunden	48 Stunden	
13 (Kontr.)	M 7 H	28		2800
»	» T	15		1500
15 (Kontr.)	M 13 RU	1		100
»	» T	39		3900
»	M 14 H	4		4000
14 (Kontr.)	M 11 F		64	6400
»	M 12 H		7	700
»	» F		4	400
»	» T		2	200

kann, wurden im Zusammenhange mit einigen der Experimente Bakterienzüchtungen aus den Inkubationslösungen vorgenommen. Die Bestimmungen wurden unter möglichst sterilen Verhältnissen in folgender Weise ausgeführt: Nach Abschliessung der Inkubation wurde die Versuchslösung mit destilliertem und sterilisiertem Wasser im Verhältnis 1: 100 verdünnt. Einem ccm dieser Lösung wurde gewöhnlicher bakteriologischer Agar in Probierröhre auf Wasserbade bei 46° C zugeführt. Der Inhalt wurde hiernach in Petri-Schälchen gegossen und in ein Thermostat bei 37° C abgestellt. (Die Agarschicht hatte eine Dicke von durchschnittlich 2—3 mm). Die Ablesungen wurden in einer Serie nach 24 und in einer anderen nach 48 Stunden bewerkstelligt, wobei die Anzahl der gesamten Bakterienkolonien festgestellt wurde. Die Resultate dieser Ablesungen sind aus der Tabelle 16 zu ersehen.

Bei der makroskopischen Untersuchung der Kolonien erwiesen sich dieselben hauptsächlich aus Staubbakterien zu bestehen, welche offenbar während der Präparierung zugekommen waren. Die annäherende Zahl der Bakterien pro ccm Inkubationslösung schwankte bei den verschiedenen Versuchen zwischen 100 und 6400. Diese geringe Anzahl dürfte dadurch erklärt werden, dass die Bestandsteile der Inkubationslösungen, ausser Spuren von im Laufe der Inkubation aus den Präparaten ausgewandertem Eiweiss,

an sich selbst einen äusserst schlechten Nährboden für Bakterien darbieten. Man dürfte wahrscheinlich sagen können, dass diese Bakterienmenge wohl kaum irgendwelche Rolle, sei es bei der Aufnahme oder bei der Retention von Calcium und Phosphor in den Präparaten gespielt haben könnte. Die Möglichkeit, dass die Bakterien Na-Glycerophosphat in Versuchen, wo dieses als Substrat zur Anwendung gekommen ist, hydrolysieren könnten, dürfte wohl auch infolge der geringen Menge der Bakterien, sowie in Anbetracht der kurzen Inkubationsdauer ausgeschlossen werden können. Diese Annahme dürfte eine weitere Stütze in der früher erwähnten Beobachtung finden, dass Lösungen von Na-Glycero-Phosphat, obwohl in nicht sterilen Verhältnissen aufbewahrt, kein Phosphor in direkt bestimmbarer Form enthielten.

E. Besprechung der Versuchsergebnisse mit normalem Knochenmaterial.

Der Zweck dieser preliminären Untersuchungen war einen Einblick in den im normalen Knochengewebe vorsichgehenden Calcium- und Phosphorstoffwechsel zu gewinnen. Bei der Beurteilung der Versuchsergebnisse scheint es angebracht allererst verschiedene Faktoren zu erwägen, welche teilweise einen Verlust von Calcium bzw. Phosphor aus der Inkubationslösung vortäuschen könnten, ohne dass eine direkte Aufnahme dieser Stoffe in die Präparate stattgefunden hätte. Die folgenden hauptsächlichen, die Entstehung von Fehlern bedingenden Faktoren, dürften hierbei in Betracht kommen.

1) Bei der Bestimmung der Calcium- und Phosphorverluste aus der Inkubationslösung ist die Gewebeflüssigkeit der Präparate unbeachtet geblieben. Es ist demnach denkbar, dass ein Teil dieser Flüssigkeit im Laufe des Versuches aus den Präparaten herausdiffundieren und dadurch vielleicht eine Verdünnung der Inkubationslösung bewirken könne. Da jedoch der Verlust der Inkubationslösung an Calcium und Phosphor in den verschiedenen Versuchen zu gross ist, um nur auf diese Weise erklärt werden zu können, scheint diese Möglichkeit keine nennenswerte Rolle spielen zu dürfen. Sollte eine solche Verdünnung der Versuchslösung

stattfinden so scheint es auch schwierig eine Erklärung dafür zu finden, dass der Phosphor nur in organischer, aber nicht in anorganischer Form aufgenommen wurde, da eine solche Verdünnung auch im letzteren Falle zu einem scheinbaren Phosphorverlust aus der Lösung führen würde. Dieselbe Motivierung ist auch für die Beurteilung der Beobachtungen über den lähmenden Einfluss des Formaldehyds auf die Absorption von Calcium und Phosphor gültig. Es muss des weiteren noch bemerkt werden, dass die Salzkonzentration der Versuchslösungen, ausser in den Serien, wo destilliertes Wasser in Anwendung gekommen ist, nahezu serumisotonisch ist, weshalb eine Diffusionsverschiebung zwischen der Gewebeflüssigkeit und der Inkubationslösung wenigstens in einer beachtenswerten Menge höchstens unwahrscheinlich ist.

2) Theoretisch kann man sich die Möglichkeit einer Bindung von Calcium und Phosphor an Zersetzungsprodukte bakterieller oder autolytischer Art in den Präparaten in einer sich dem Nachweis in der Suspensionslösung entziehenden Form vorstellen. Die geringe Menge der in der Versuchslösung nach abgeschlossener Inkubation vorkommenden Bakterien lässt jedoch eine beachtenswerte bakterielle Zersetzung der Präparate als unwahrscheinlich vorkommen. Eine Bindung durch Zersetzungsprodukte dürfte auch die relativ gleichmässigen Werte des aufgenommenen Calcium und Phosphor in den verschiedenen Serien sowie den deutlichen Unterschied in der Adsorption der verschiedenen Phosphorfraktionen kaum erklären können. Es ist schwer genau zu beurteilen, wie lange das Knochengewebe sich im Versuchs-Milieu überlebt oder, in anderen Worten, wie lange dasselbe während der Inkubation seine physiologischen Funktionen behält. Man dürfte jedoch damit rechnen können, dass diese Zeit von verhältnismässig langer Dauer ist. Shipley, Kramer und Howland geben z. B. in ihren Versuchen in vitro auf, dass eine histologisch nachweisbare Calcifikation des Epiphysenknorpels von Ratten nach einer Inkubationszeit von 2 Wochen bei 37° Temperatur noch verspürt werden konnte. Mit Ausnahme der etwas niedrigeren Temperatur sind die Versuchsbedingungen bei meinen Experimenten im grossen ganzen dieselben wie bei denen der obenerwähnten Forscher gewesen. Es wäre jedoch angebracht, einen Unterschied im Vergleich mit den früher im historischen Übersicht erwähnten

Versuchen in vitro hervorzuheben. Während bei diesen entweder dünne Knochenscheiben oder fein zerteiltes Knochengewebe als Versuchsmaterial zur Anwendung kamen, sind in meinen Experimenten, mit Ausnahme von den Versuchen mit Knorpelgewebe, die Knochenpräparate intakt gewesen, und solche Präparate besitzen wahrscheinlich auch ein grösseres Vermögen ihre physiologischen Funktionen zu bewahren, als in den Fällen, wo sie einer vorherigen mechanischen Behandlung unterworfen werden.

Verschiedene Umstände sprechen dafür, dass die Adsorption von Calcium und Phosphor schon in nahem Anschlusse an die Inkubation beginnt. Robison z. B. hat bei seinen Versuchen Zeichen der Verkalkung von Knorpelpräparaten schon 4 Stunden nach Anfang der Inkubation beobachten können.

3) Es verbleibt als Hauptsache zu erwägen, ob Calcium von aus den Präparaten diffundiertem Phosphor gefällt werden könnte, und in Übereinstimmung hiermit dürfte auch der Gedanke nahe liegen, dass in Versuchen, in denen Phosphat als Substrat angewandt worden ist, ausgewandertes Calcium Phosphor ausfällen könne. Diese Fehlermöglichkeit darf nicht unbeachtet gelassen werden, insbesondere da aus den Versuchen hervorgeht, dass in calcium- und phosphorfreien Lösungen inkubierte Präparate diese recht bedeutende Mengen von Calcium und Phosphor abgaben. Eine nähere Begründung dürfte jedoch dem widerreden, dass eine wesentliche Verminderung des Calcium- und Phosphorgehaltes in der Versuchslösung von einer solchen Fällung herrühren könne. In der Mehrzahl der Experimente konnte nach Abschluss der Inkubation keine Fällung in den Versuchslösungen wahrgenommen werden. Eine in einigen Lösungen aufgetretene schwache homogene Opaleszenz wurde durch aus den Präparaten heraus diffundiertes Eiweis erklärt. Ein wiegender, einer nennenswerten Fällung von Calciumphosphat in Inkubationslösungen widersprechender Umstand ist die Beobachtung, dass Phosphor nur in organischer Form in die Präparate aufgenommen wurde. Sollte eine Fällung von Calciumphosphat stattfinden, dürfte dieses ja einen scheinbaren Verlust von anorganischem Phosphat in der Inkubationslösung mit sich führen. Bei Versuchen in Reagenzgläsern erwies es sich nämlich, dass beim Zusatz von Phosphat in eine Calciumchlorid-Lösung eine Fällung beträchtlich schneller und bei einer niedri-

geren Phosphorkonzentration vorsichging in dem Falle, wo der Phosphor in anorganischer und nicht in organischer Form vorlag.

Von einer nennenswerten Fällung von Calcium und Phosphor in Inkubationslösungen durch andere aus den Präparaten diffunddierte Stoffe kann wohl kaum die Rede sein. Diese Vermutung wird von PFÄUNDLERS Untersuchungen experimentell unterstützt, in denen es ihm nicht gelungen war, durch Extrahieren von Knochenmasse vermittelst schwacher Base oder Säure ein Filtrat zu gewinnen, das aus einer 0.1 n Calciumchloridlösung in vitro Calcium fällen konnte.

Es steht also fest, dass die aus den Inkubationslösungen im Laufe der Versuche verschwundenen Calcium und organischer Phosphor wenigstens zum überwiegenden Teil in die Präparate aufgenommen worden sind. Es entsteht hierbei die Frage: Welcher Mechanismus liegt der Adsorption von Calcium und organischem Phosphor in das Knochengewebe zu Grunde? Im Lichte der dualistischen Auffassung des Verknöcherungsprozesses kann man mit zweien hauptsächlichen Möglichkeiten rechnen. Die erste, welche die einfachste Erklärung der Adsorptionserscheinung darbieten dürfte, würde dieselbe als ein einfaches Diffusions- oder Imprägnierungsphänomen betrachten, bei dem das Knochengewebe selbst eine mehr oder weniger passive Rolle spielen würde. Andererseits liegt die Möglichkeit vor, dass die Aufnahme von Calcium und Phosphor der Ausdruck einer vitalen und aktiven Funktion des Knochengewebes wäre. Im Zusammenhange mit der Beurteilung der verschiedenen Versuchsergebnisse werden im folgenden diese beiden Möglichkeiten einer näheren Erörterung unterworfen.

Es wurde in der Einleitung beobachtet, dass die Knochen aus einer umgebenden Lösung Calcium aufnahmen. Es muss bemerkt werden, dass die initiale Konzentration von Calcium in der Inkubationslösung den Calciumspiegel des Serums nicht überstiegen hat. HELVE (1941) gibt diesen als durchschnittlich 12.6 mg % Ca in normalen Ratten auf. Kein von einem Überschreiten der serumisotonischen Verhältnisse abhängiges Diffusionssystem liegt also der Adsorption zu Grunde, insbesondere wenn man beachtet, dass Calcium auch aus destilliertem Wasser aufgenommen wurde. Es scheint also als ob das Knochengewebe ein aktives Vermögen Calcium aufzunehmen besitze. Dabei dürfte das Ver-

muten nahe liegen, dass die Calcium-Affinität sich allererst auf die dissoziierte Calciumfraktion einrichtet. Da, wie bekannt, ein grosser Teil des Serumcalciums an Protein gebunden ist, scheint eine gleichartige Fixation von Calcium an Proteinsubstanzen im Knochengewebe denkbar zu sein. Dieses dürfte nicht ohne Interesse für die Ossifikation sein, da hierdurch eine primäre Aufnahme von Calcium ermöglicht wäre, ohne dass eine lokale Übersättigung der Calciumverbindungen im Serum in dem Sinne, dass eine spontane Fällung von Calciumsalz in der Knochensubstanz vorsichgeht, eine Voraussetzung hierfür sein müsse. Diese Annahme steht in gutem Einklang mit der »elektiven Ossifikations-Theorie» von PFAUNDLER, FREUDENBERG und GYÖRGY.

Betreffend die calciumbindende Substanz des Knochengewebes können verschiedene Möglichkeiten in Frage kommen. PFAUNDLER, z. B., stellt sich vor, dass die Osseoalbumoiden die Rolle von »Kalkfängern» spielen dürften. RABL dagegen schreibt diese Bedeutung den Aminoverbindungen zu. Andererseits dürfte man annehmen, dass auch der Kollagen oder Osseomucoid des Knochengewebes die Rolle von calciumbindenden Faktoren spielen könnten. Man dürfte vielleicht das physiologische Dasein eines bewegbaren Verschiebungssystems zwischen dem diffusiblen Serumcalcium, dem Serumprotein und den Proteinsubstanzen des Knochengewebes annehmen können. Die Aufnahme von Calcium in Knochen könnte vielleicht auf die höhere Calcium-Affinität der Knochenproteine den Serumproteinen gegenüber zurückgeführt werden. Verschiedene Umstände sprechen für die Existenz eines solchen »Seilziehens» zwischen verschiedenen Proteinsubstanzen. FREUDENBERG und GYÖRGY beobachteten somit in ihren Versuchen, dass Gehirn-Substanz, im Gegensatz zum Knochengewebe, Calcium aus Serum-Lösungen nicht aufnahm, welches Phänomen sie dadurch erklären, dass die Serumproteinen eine grössere Affinität dem Calcium gegenüber als die Gewebekolloiden der Gehirnsubstanz besitzen. Es können noch in diesem Zusammenhange die histologischen Beobachtungen von SHIPLEY, KRAMER und HOWLAND angeführt werden, dass ein Zusatz von 2 % »egg albumin» in einer Calcium und Phosphor enthaltenden Salzlösung die Calcifikation des Epiphysenknorpels von Ratten hemmt. Wurde das Präparat nach Kontakt mit Protein in einer von diesem Stoffe freien Ver-

suchslösung inkubiert, fand die Calcifikation in der gewöhnlichen Weise statt, welches zeigt, dass die vom Protein auf die Calcifikation ausgeübte hemmende Wirkung nicht auf irgendeine Schädigung des Knorpelgewebes selbst zurückgeführt werden konnte.

Der Umstand, dass eine Aufnahme von Calcium bei einer so niedrigen Konzentration, wie in den bei den Versuchen angewandten Inkubationslösungen, (10—11 mg % Ca), stattfand, steht in einem scheinbaren Widerspruch zu den histologischen Wahrnehmungen von SHIPLEY und ROBISON. Diese Forscher zeigten, wie früher erwähnt, dass eine Voraussetzung für die Calcifikation von Epiphysenpräparaten in vitro der Umstand ist, dass das Produkt der Calcium- und Phosphormenge der Inkubationslösung 30 mg % übersteigen muss. Als eine Erklärung dieses scheinbaren Widerspruchs muss jedoch hervorgehoben werden, dass die Aufnahme von Calcium und Phosphor ins Knorpelgewebe eine Ossifikation in der Form einer Ausfällung von Calciumphosphat voraussetzt um histologisch nachgewiesen werden zu können. Dagegen kann eine Adsorbtion von nur Calcium oder Phosphor dem histologischen Nachweise sich wahrscheinlich entziehen. Hierdurch tritt auch der grundsätzliche Unterschied zwischen einer primären Adsorbtion von Calcium und Phosphor und der endgültigen Ossifikation in deutlicher Beleuchtung hervor.

Die Beobachtung, dass das Knochengewebe Calcium in recht bedeutender Menge an eine calciumfreie physiologische Salzlösung abgab, ist vielleicht einer näheren Berücksichtigung wert. Der Versuch legt mit aller erwünschten Deutlichkeit dar, dass wenigstens ein Teil des Kalkes im Knochengewebe nicht stabil fixiert ist. Bei der Beurteilung, auf welche Weise das Herausdiffundieren von Calcium zu stande kommt, kann man sich vorstellen, dass ein Teil des Calciums aus der interstitiellen Gewebelymphe herstammt. In Anbetracht der Winzigkeit der Präparate dürfte es jedoch als äusserst unwahrscheinlich erscheinen, dass dieser Mechanismus eine nennenswerte Rolle spielen könnte. Eine Mobilisierung von Calcium in der Form von Komplexverbindungen in der Knochen-Substanz dürfte dagegen nahe liegen. Es ist nicht ausgeschlossen, dass die Citronensäure teilweise als eine Komponente solcher Komplexverbindungen dienen könnte. Andererseits kann man,

ausgehend von der Annahme, dass ein Teil des Calciums an Proteinsubstanzen im Knochengewebe gebunden ist, sich die Möglichkeit einer Abspaltung von Calcium aus einer solchen labilen Verbindung vorstellen. Die Fixation des Calciums mit dem Protein des Knochengewebes dürfte in einem solchen Falle als ein reversibler Prozess betrachtet werden können. Es fällt schwer endgültig zu entscheiden, welcher Mechanismus der Decalcifikation des Knochen-Gewebes zu grunde liegen könne, aber es lässt sich denken, dass er zum Teil in irgendeinem Zusammenhange mit der salzauflösenden Tätigkeit der Osteoklasten im Knochengewebe steht.

Bei den Versuchen wurde die bemerkenswerte Beobachtung gemacht, dass normales Knochengewebe das Vermögen aus einer Inkubationslösung anorganischen Phosphor in der Form von sekundärem Natriumphosphat bei einer initalen Phosphorkonzentration von bis auf 50 mg % aufzunehmen nicht zu besitzen scheint. Lag dagegen der Phosphor in organisch gebundener Form, wie Na-Glycerophosphat vor, so fand eine deutliche Aufnahme von Phosphor in die Präparate statt. Bei den Versuchen mit Meerschweinchen-Knochen wies in diesem Falle der adsorbierte Phosphor durchschnittliche Werte von etwa 20 % der in der Inkubationslösung anfänglich enthaltenen Phosphormenge auf. Dieser prägnante Unterschied in der Aufnahme dieser zweien Phosphorfraktionen scheint die Möglichkeit auszuschliessen, dass bloss ein einfacher physikalischer Diffusions-oder »Imprägnierungs»-Prozess der Adsorption zu grunde liegt. Der Mechanismus scheint viel komplizierter zu sein. Eine anorganische Phosphorkonzentration von 50 mg % in der Inkubationslösung übersteigt bedeutend den anorganischen Phosphorgehalt im Serum, weshalb man in diesem Falle von einer direkten Übersättigung an Phosphor im Verhältnis zu den im Serum herrschenden physiologischen Umständen sprechen darf. Es fällt schwer festzustellen, weshalb anorganischer Phosphor dessenungeachtet von den Präparaten nicht aufgenommen wurde. Diese Wahrnehmung bestätigt zum Teil die histologischen Untersuchungen Robisons, in denen er zeigte, dass Epiphysenknorpel von Ratten viel schneller und bei einer niedrigeren Phosphorkonzentration in Calcium und Phosphat enthaltenden Inkubationslösungen calcifiziert wurde in dem Falle, wo das letzterwähnte Substrat in der Form von Glycerophosphat und nicht von anorganischem Phos-

phor vorlag. Um eine Calcifikation im letzteren Falle hervorzurufen, musste die Inkubationslösung mit Calciumphosphat übersättigt sein.

Die Wahrnehmung von FREUDENBERG und GYÖRGY, dass das Knochengewebe, um in vitro anorganisches Phosphat aufnehmen zu können, primär mit Calcium angereichert sein musste, gibt eine Erklärung der augenscheinlichen Unfähigkeit des Knochen-Gewebes anorganisches Phosphat bei der in meinen Versuchen für die Inkubationslösungen angewandten Phosphorkonzentration primär adsorbieren zu können. Es wäre jedoch angebracht in diesem Zusammenhange eine Mitteilung von ULLRICH (1929) hervorzuheben, nach welcher Knochengewebe unter gewissen Bedingungen anorganischen Phosphor in vitro verwerten kann. Bei seinen Versuchen digerierte er feinverteilte Knochensubstanz in 0.5—1 % anorganisches Phosphat enthaltenden Lösungen, wobei er einen Phosphorverlust aus der Inkubationslösung feststellen konnte. Es muss jedoch bemerkt werden, dass die Versuchsbedingungen bei diesen Experimenten von den im normalen Serum herrschenden physiologischen Verhältnissen ansehnlich abweichen.

Weshalb wurde denn estergebundener Phosphor vom Knochen-Gewebe begierig adsorbiert? Betrachtet man dieses Phänomen im Lichte der Untersuchungen ROBISONS, so erscheint es als annehmbar, dass die Adsorption teilweise im Zusammenhange mit der Phosphatasetätigkeit im Knochengewebe stehe. Man könnte sich vielleicht vorstellen, dass eine *primäre* Fixation der Phosphorsäureestern an diese Enzymen eine Voraussetzung dafür wäre, dass eine hydrolysierende Wirkung der Phosphatasen zu stande kommen würde. Hierbei liegt die Vermutung nahe, dass die Phosphatase eine aktive Affinität für organisch gebundenen Phosphor besitzen. Dieses würde in seiner Reihe erklären, weshalb das Knochengewebe aus einer Inkubationslösung estergebundenen Phosphor an sich zog, wogegen eine ähnliche Erscheinung ausblieb, wenn anorganischer Phosphor als Substrat angewandt wurde. Da die Phosphatasen, wie von ROBISON dargelegt, in den Knochen-Zellen gebildet werden, dürfte die Adsorption von Phosphorsäure-Estern als der Ausdruck einer vitalen Aktivität des Knochengewebes aufzufassen sein. Ausgehend von dieser Annahme, müsse diese Aufnahme von Phosphat in der unmittelbaren Nähe der Knochen-Zellen am stärksten sein.

Da, wie bekannt, auch Serum Phosphatasen enthält, scheint es als ob diese Enzymen im Knochengewebe nicht stabil fixiert wären. Bei mit organischem Phosphat als Substrat ausgeführten Versuchen lag nach abgeschlossener Inkubation etwa die Hälfte der initialen Phosphorfraktion in anorganischer Form vor. Dieses kann teilweise als ein Ergebnis der hydrolysierenden Wirkung der im Laufe des Versuches aus den Präparaten ausdiffundierten Phosphatasen erklärt werden. Einem solchen Mechanismus darf jedoch keine entscheidende Rolle zugeschrieben werden, da die Versuchsresultate als mehr wahrscheinlich erscheinen lassen, dass ein grosser Teil dieses anorganischen Phosphors den Knochen entstammte. Es wurde nämlich in den Versuchen festgestellt, dass das Knochengewebe an eine phosphorfreie physiologische Salzlösung anorganisches Phosphor in ziemlich bedeutender Menge abgab. Es erscheint folgentlich offenbar, dass wenigstens ein Teil des Phosphors im Knochengewebe, ebenso wie es mit Calcium der Fall ist, nicht stabil fixiert ist. In Ansicht der Bedeutung des Knochengewebes als Mineraldepot, ist die augenscheinliche Leichtigkeit, mit welcher Calcium und Phosphor aus demselben mobilisiert werden zu können scheinen, nicht uninteressant, und scheint, was die Art der ausdiffundierten Phosphorfraktion betrifft, wenigstens eine teilweise Verbindung mit Calcium wahrscheinlich. Andererseits könnte man sich auch vorstellen, dass der in die Inkubationslösung ausgewanderte Phosphor zum Teil mit der Phosphataseaktivität im Knochengewebe im Zusammenhange stehen könnte. Der Schlusseffekt der hydrolysierenden Tätigkeit dieser Enzymen dürfte nämlich vielleicht zu einem Überschuss an freidiffusiblem anorganischen Phosphat in der Knochensubstanz führen können.

Aus den Versuchen ist ersichtlich, dass von den verschiedenen Teilen eines und desselben Knochens die Epiphysenknorpeln annäherend doppelt so viel Calcium und Phosphor wie die Knochen-Diaphyse adsorbierter. Da der Zuwachs in dieser letzteren Zone am stärksten ist, scheint diese Wahrnehmung von rein physiologischem Standpunkte aus logisch zu sein. Die Verteilung von Calcium und Phosphor im Knorpel, bzw. Knochengewebe, zeigt, dass Knorpel-Teile um zu verknöchern, bedeutende Mengen Calcium und Phosphor aufnehmen müssen. Bei der Analyse verschiedener Extremitäts-

Knochen von Feten geben SWANSON und IOB (1937) einen Calcium-Gehalt von 7.9 Millimol auf je 100 g Trockensubstanz in dem Epiphysenknorpel gegen 514.8 Millimol in den Diaphysen auf. Die entsprechenden Phosphorwerte waren 8.6, bzw. 336.4 Millimol. Es liegt also die Vermutung nahe, dass die Epiphysen im Vergleich mit der Diaphyse besonders Calcium-und Phosphor-»hungrig» sind. Welcher Mechanismus dürfte denn der starken Begierde des Knorpelgewebes nach diesen Stoffen zu grunde liegen? Was, erstens, die Aufnahme von Calcium betrifft, so kann man, ausgehend von der Annahme, dass wenigstens ein Teil desselben an Proteinsubstanzen adsorbiert wird, sich vorstellen, dass diese Stoffe infolge von qualitativen oder quantitativen Verschiedenheiten Calcium mit ungleicher Stärke adsorbieren. Der Erweis von ROBISON und ROSENHEIM (1934), dass Knorpelgewebe bedeutend schneller als Organe, wie z. B. Nieren und Lunge in vitro calcifiziert wurde, dürfte möglicherweise von einem gleichen Kausal-Moment abhängen.

Sollte man die Aufnahme von organischem Phosphor in Verbindung mit der Phosphatasetätigkeit in den Präparaten stellen, so kann die Wahrnehmung, dass der Epiphysenknorpel mehr Phosphor als die Diaphyse aufnahm, vielleicht eine einleuchtende Erklärung finden. Aus den von FELL und ROBISON (1929) ausgeführten Untersuchungen geht nämlich hervor, dass die Epiphysen bedeutend mehr Phosphatase als die Diaphyse enthalten.

Ausser organischem Phosphor (2.9 mg %) enthielt die Inkubationslösung anfänglich auch anorganisches Phosphor (4.4 mg %), weshalb man mit in Rechnung ziehen muss, dass der adsorbierte Phosphor auch von dieser letzteren Fraktion herstammen konnte. Im Kenntnis, dass anorganisches Phosphat in Versuchen mit Meerschweinchen in die Knochen nicht aufgenommen wurde, erscheint es doch glaublich, dass auch in diesem Falle die Adsorption vor allem dem organisch gebundenen Phosphor galt. Diese Annahme findet zum Teil eine Stütze in der Beobachtung, dass, mit Ausnahme eines einzigen Falles, die Phosphorfraktion der Inkubationslösungen nach Abschluss des Versuches keinen Phosphor in estergebundener Form enthielt.

Es ist bemerkenswert, dass die Epiphysen, die Calcium und Phosphor besser als die Diaphyse adsorbierten, diese Stoffe auch

stärker als der Knochenteil fixierten. Von osteogenetischem Gesichtspunkte aus dürfte diese Beobachtung vielleicht nicht ohne Interesse sein, da sie den Calcium- und Phosphor-»Hunger» des Knorpelgewebes so zu sagen in doppelter Bemerkung zum Vorschein kommen lässt. Schwierigkeiten stellen sich dagegen ein bei der Beurteilung, was für ein primärer Anlass dazu zu grunde liegen könnte, dass der Epiphysenteil an eine Basallösung weniger Calcium und Phosphor als die Diaphyse abgiebt. Es ist aber hypothetisch annehmbar, dass der Unterschied in der Retention von Calcium einigermassen in einem ungleich starken Vermögen der Gewebeproteinen Calcium zu fixieren zu suchen wäre, welche Eigenschaft zu dem Vermögen diese Stoffe zu adsorbieren proportional sein dürfte. Andererseits liegt auch die Möglichkeit vor, dass die grössere Stabilität der Epiphysen den Komplexverbindungen von Calcium und Phosphor gilt in Anbetracht dessen, dass auch der Phosphor stärker als in der Diaphyse fixiert wurde.

Die Beobachtung, dass Formaldehyd auf das Vermögen der Knochen Calcium und organisches Phosphat zu adsorbieren lähmend wirkte, dürfte nicht ohne Interesse sein. Da, wie bekannt, Formaldehyd ein starkes Zellengift ist, zeigt der Versuch deutlich, dass der Adsorptionsmechanismus mit aktiven biochemischen Prozessen des Knochengewebes eng verbunden ist. Diese Versuche bestätigen auf chemischem Wege die histologischen Wahrnehmungen von Shipley und Robison, dass Formaldehyd die Calcification von Epiphysenknorpel in vitro inhibiert. Diese Forscher bedienten sich aber einer ansehnlich grösseren Konzentration des Formaldehyds (10 %) in ihren Inkubationslösungen.

Bei der Auslegung meiner Ergebnisse muss beachtet werden, dass der pH der Inkubationslösung vielleicht vom Folmaldehyd einigermassen verändert wird. Dieser Umstand, in Anbetracht der von Robison gemachten Beobachtung, dass kleinere Verschiebungen im pH der Inkubationslösung keine Wirkung auf die Calcifikation in vitro ausüben, dürfte jedoch kaum allein den Verschiedenheiten in der Absorption zu grunde liegen.

Sollte man von der Annahme ausgehen, dass der Adsorption von Calcium und Phosphor in den Knochen enzymatische Prozesse zu grunde liegen, so erscheint es als wahrscheinlich, dass der Effekt des Formaldehyds auf eine Lähmung der Wirksamkeit dieser

Enzymen zurückzuführen ist. Hinsi htlich der Knochenphosphatase geben ROBISON, MACLEOD und ROSENHEIM im Anschluss an ihre Versuche auf, dass dieselben von Formaldehyd verhältnissmässig leicht beeinflusst werden. 1 % Formaldehyd verminderte somit die Phosphatasewirksamkeit mit 25 %, wogegen 10 % Formaldehyd den hydrolysierenden Effekt dieser Enzymen völlig inhibierte. Wenn wir von dieser Beobachtung ausgehen, so dürfte das Vermuten nahe liegen, dass Formaldehyd die Phosphatase des primären Vermögens beraubt ihre Substrate zu adsorbieren. Diese Annahme würde teilweise eine Erklärung des Umstandes darbieten, dass beim Anwesen von Formaldehyd Natriumglycerophosphat in meinen Versuchen in die Knochen nicht aufgenommen wurde.

Es wurde früher die Vermutung ausgesprochen, dass organisches Phosphat in Inkubationslösungen teilweise von den aus den Präparaten herausdiffundierten Phosphatasen hydrolysiert werden könnte. Der Umstand, dass die finale anorganische Phosphorfraktion in Inkubationslösungen in der Anwesenheit von Formaldehyd im Durchschnitt nur etwa 10 % des gesamten saurelöslichen Phosphors betrug, wogegen dieser Wert in den Kontrollserien etwa 35 % war, dürfte im Kenntnis des phosphatasehemmenden Effekts des Formaldehyds im guten Einklange mit einer solchen Annahme stehen.

Durch ihre Vergiftungsversuche stellten SHIPLEY, KRAMER und HOWLAND fest, dass kleine Mengen von Toluol die Calcifikation in vitro bloss teilweise verhinderten. Nur in einem Falle konnten sie eine völlige Inhibierung der Aufnahme von Calcium und Phosphor in Knorpelpräparaten beobachten. Meine Versuche bestätigen zum Teil diese Ergebnisse. Der Umstand, dass Toluol die Fähigkeit der Knochen organisches Phosphat zu adsorbieren nicht gänzlich lähmte, obwohl die Aufnahme im Anwesen dieser Agens dennoch bedeutend schwächer als in den Kontollversuchen war, dürfte wahrscheinlich davon abhängen, dass das Toluol in der bei den Versuchen angewandten Konzentration keinen gleichen toxischen Effekt auf biochemische Prozesse im Knochengewebe wie der Formaldehyd ausübt.

Die oben erörterten Ergebnisse haben dargelegt, dass die Adsorption und das Fixieren von Calcium und Phosphor im Knochengewebe in einem engen Verhältnis zu einander zu stehen

scheinen. Einen weiteren Beleg dafür ergibt die Beobachtung, dass der Formaldehyd, indem er die Knochen der Fähigkeit beraubte Calcium und Phosphor aufzunehmen, gleichzeitig auch die Fixierung dieser Stoffe in den Knochen schwächte. Dieser letztere Effekt äusserte sich dadurch, dass Calcium und Phosphor beim Anwesen dieser Agens in einer basalen Salzlösung aus den Knochen bedeutend mehr als in der Kontrollserie »leckten». Es ist deshalb wahrscheinlich, dass die Fähigkeit der Knochen Calcium und Phosphor sowohl zu adsorbieren, wie auch zu fixieren mit einem aktiven Prozess verglichen werden kann. Es ist bemerkenswert, dass beim Anwesen von Formaldehyd nur ungefähr die Hälfte des ausdiffundierten Phosphors in anorganischer Form vorlag, wogegen bei den Kontrollversuchen beinahe die gesamte ausgewanderte Phosphormenge von dieser Phosphorfraktion vertreten war. Man kann wohl diesen Umstand einigermassen als einen Ausdruck dafür deuten, dass der Formaldehyd die Phosphatasetätigkeit in den Knochen vermindert hatte.

Es erscheint als angebracht noch eine kurze Beurteilung darüber anzustellen, in welchem Masse diese in vitro erhaltenen Ergebnisse für die im lebenden Organismus waltenden Verhältnisse verwertet werden können. Ein gewisser, für das Berechtigte eines solchen Vergleichs mit den physiologischen Verhältnissen sprechender Umstand ist die oben erwähnte Beobachtung von Shipley und Robison darüber, dass Epiphysenknorpel, was sein histologisches Bild betrifft, auf genau dieselbe Weise in vitro, wie unter den physiologischen Verhältnissen in vivo calcifiziert wurde. Es muss jedoch in Betracht gezogen werden, dass in vitro die Bedingungen in vielen Hinsichten ungünstiger als im lebenden Organismus sind. Die Calcium- und Phosphorspannung nimmt im Reagenzglase im Laufe des Versuches allmählich ab und ein etwaiger begünstigender Einfluss anderer Ionen und Nicht-Elektrolyten in der in der Gewebeflüssigkeit vorfindlichen Form wird in meinen Versuchen eliminiert. Ausserdem schafft wahrscheinlich die Zimmertemperatur nicht ebenso vorteilhafte Voraussetzungen für biochemische Prozesse wie die Temperatur des Körpers. Der Mangel an Protein in der Inkubationslösung bedeutet eine wesentliche Abweichung von den physiologischen Verhältnissen. Dieses kann insbesondere für die Adsorption von Calcium in Knochen von Bedeutung sein.

Da ein grosser Teil des Calciums im Serum mit Protein gebunden ist, fällt nämlich in den Versuchen ein so zu sagen »konkurrierender» calciumbindender Faktor weg. Diese Annahme findet eine Stütze in der vorstehend erwähnten Beobachtung von SHIPLEY, KRAMER und HOWLAND, dass der Inkubationslösung zugeführtes Protein die Calcifikation von Epiphysenknorpel in vitro hemmt. ROSENHEIM (1934) spricht von demselben Effekt bei analogen Versuchen beim Anwesen von 7 % Pferdeserumprotein in der Versuchslösung.

Es wäre jedoch angebracht in diesem Zusammenhange zu betonen, dass unser Kenntnis von der Zusammensetzung der interstitiellen Gewebeflüssigkeit der Knochen bei weitem nicht vollständig ist. Dieses Kenntnis ist jedoch eine unumgängliche Voraussetzung, um den Calcium- und Phosphorstoffwechsel im Knochengewebe eingehend beurteilen zu können. Bei meinen Versuchen entsprach die Calciumkonzentration der Inkubationslösung nahezu dem Calciumgehalt des normalen Serums. Die Phosphormenge ist dagegen in den meisten Versuchen etwas grösser als der totale Phosphorgehalt im Serum, der beim Mensch, wie bekannt, etwa 7—12 % ist (nach ANNERSTEN 1940). Es muss jedoch beachtet werden, dass Vollblut einen bedeutend höheren Phosphorspiegel als das Serum hat. HELVE (1940) gibt für normale Ratten einen Durchschnittswert von 29.3 mg% des gesamten säurelöslichen Phosphors im Blute auf, von welchen 24 mg % in estergebundener Form vorliegen. Der in den Korpuskeln deponierte Phosphor scheint in das Plasma leicht abgegeben werden zu können. SHIPLEY, KRAMER und HOWLAND (1926) geben z. B. auf, dass falls man Blut bei 37° Temperatur eine Zeit lang stehen lässt, so erhöht sich die Menge des anorganischen Phosphors im Serum. In mit Rattenblut angestellten Versuchen wuchs nach 24 Stunden der Gehalt an anorganischem Serumphosphor, der normal etwa 5 mg % war, in einigen Fällen bis auf 20 mg%. Im Falle man Knochenextrakt auf Vollblut einwirken lässt, steigt auch, wie ROBISON dargelegt hat, der Serumphosphor ansehnlich. Diese Versuche sind insofern interessant, dass sie die Leichtigkeit darlegen, mit der der Phosphor bei Bedarf aus den Korpuskeln, insbesondere in der Nähe des Knochengewebes, mobilisiert werden kann. Unter solchen Verhältnissen ist es nicht ausgeschlossen, dass die Gewebeflüssigkeit der Knochensubstanz ansehnlich mehr

Phosphat als das Serum enthält. Die Phosphatkonzentration der Inkubationslösung braucht deshalb in meinen Versuchen den entsprechenden Spiegel der interstitiellen Gewebeflüssigkeit nicht zu übersteigen.

Sollte man auf Grund der Experimente versuchen sich eine Auffassung über den normalen Ossifikationsmechanismus zu bilden, so erscheint es als berechtigt, in demselben zwischen einer primären Adsorption von Calcium und Phosphor im Knochen-Gewebe und einer definitiven Fällung von Knochensalz in dessen Schlussform zu unterscheiden. Der ersterwähnte Prozess dürfte s. z. s. die Einleitung der Ossifikation bilden. Die Aufnahme von Calcium und Phosphor in das Knochengewebe darf jedoch nicht als ein isoliertes Phänomen, sondern eher als ein Teilprozess eines und desselben Systems betrachtet werden. Die Korrelation zwischen dem Calcium- und Phosphorstoffwechsel im Knochengewebe geht u. a. aus der Beobachtung FREUDENBERG und GYÖRGYS hervor, dass Phosphat in vitro die Bindung von Calcium in der Knochen-Substanz befördert. ROBISON seinerseits schreibt dem organischen Phosphorradikal die Bedeutung eines Katalysators für enzymatische Prozesse im Knochengewebe zu, deren Wirkungen auch mit dem Calciummetabolismus in enger Verbindung stehen. Andererseits scheint, wie früher erwähnt, das Anwesen von Calcium von grosser Bedeutung für die Aufnahme von Phosphat in die Knochen-Substanz zu sein. Unter solchen Verhältnissen ist es schwer zu entscheiden, was für ein Prozess die s. z. s. einleitende Phase der Ossifikation ist. Im Kenntnis der besonderen Affinität des Knochen-Gewebes für Calcium, liegt dennoch die Möglichkeit vor, dass dieser Stoff primär fixiert wird, was eine spätere Aufnahme von Phosphat befördern würde. Zu Gunsten einer solchen Annahme dürfte man noch die Beobachtungen von SCHWARZ, EDEN und HERMANN (1924) erwähnen, nach welchen Callusgewebe in seinem frühen Stadium einen Überschuss an Calcium im Vergleich mit normaler Knochen-Substanz enthält.

Andererseits erscheint es nicht ausgeschlossen, dass das Knochengewebe primär aus dem Serum bzw. der interstitiellen Gewebeflüssigkeit — gemäss den in der Übersicht beschriebenen Theorien — Calcium und Phosphor in komplexer Form aufnehmen könnte. Es muss jedoch beachtet werden, dass diese beiden Auf-

nahmemechanismen einander nicht auszuschliessen brauchen. Es ist dennoch nicht gänzlich ausgeschlossen, dass man bei der Aufnahme von Calcium und Phosphor ins Knochengewebe mit einer Adsorption sowohl von diesen Ionen, wie auch von Komplexverbindungen rechnen kann.

F. Aufnahme und Retention von Calcium und Phosphor im Knochengewebe bei D-Hypervitaminose.

Die oben beschriebene in vitro-Technik bietet uns eine Möglichkeit den Calcium- und Phosphorstoffwechsel im Knochen-Gewebe unter verschiedenen Bedingungen zu studieren. Um bei diesen Versuchen, in denen der Einfluss des Vitamins D untersucht wurde, Voraussetzungen für vergleichende Schlussfolgerungen zu schaffen, ist das Tiermaterial für die Kontroll- und Parallelserien unter Beachtung der grossmöglichsten Gleichförmigkeit des Gewichtes der Knochenpräparate gewählt worden. Alle bei diesen Versuchen in Anwendung gekommenen Tiere waren mit einigen wenigen Ausnahmen männlichen Geschlechts. Die Tiere wurden am nächsten Tage nach Beendigung der Zuführung von Vigantol bzw. Sesamöl getötet. Bei diesen Versuchen kamen insgesamt 212 Präparate (97 Kontroll) von 27 Ratten (15 Kontrolltiere) und 13 Meerschwein chen (6 Kontrolltiere) zur Anwendung. Das Inkubationsmilieu der Präparate von Kontroll- und D-hypervitaminotischen Tieren in den verschiedenen Parallelversuchen ist, um den Effekt des D-Vitamins so ungestört wie möglich zum Vorschein kommen zu lassen, genau gleich gewesen.

1. *Aufnahme von Calcium.*

In den nachstehenden Versuchen wurde der Einfluss von Vitamin D auf die Aufnahme von Calcium in 33 Vorder- und Hinterbeinspräparaten von 9 Ratten studiert. Das Kontrollmaterial umfasste 20 Präparate von 6 Tieren. In den verschiedenen Versuchen wurde die Vitaminisierung im Laufe von 3—4 Tagen vorgenommen und die Tagesdosen schwankten zwischen 12000 und 36000 I. E. Vitamin D. Der Calciumgehalt der Inkubationslösung betrug in sämtlichen Fällen 10.8 mg %.

TABELLE 17. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)
Kontrolle

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 14	1.0 × 3	HRU	500	10.8	9.1	0.17
		HRU	500	10.8	9.6	0.12
		FT	950	10.8	9.1	0.17
		FT	950	10.8	9.6	0.12
R 15	1.0 × 4	RU	400	10.8	10.3	0.05
		F	1290	10.8	10.2	0.06
R 16	1.0 × 4	F	1250	10.8	10.6	0.02
		F	1240	10.8	10.6	0.02
R 17	2.0 × 3	HRU	720	10.8	10.2	0.06
		HRU	720	10.8	10.6	0.02
		FT	1100	10.8	9.8	0.10
		FT	1110	10.8	9.7	0.11
R 18	2.0 × 3	HRU	600	10.8	10.0	0.08
		HRU	625	10.8	10.5	0.03
		FT	990	10.8	9.8	0.10
		FT	1030	10.8	10.1	0.07
R 19	3.0 × 3	HRU	740	10.8	10.2	0.06
		HRU	890	10.8	11.0	– 0.02
		FT	1330	10.8	10.4	0.04
		FT	1600	10.8	10.3	0.05
Durchschnittswert:						
Vorderbeine			**633**	**10.8**	**10.2**	**0.06**
Hinterbeine			**1167**	**10.8**	**10.0**	**0.08**

Ein Vergleich zwischen den Ergebnissen der in den Tabellen 17 und 18 angeführten Versuche zeigt, dass das D-Vitamin die Fähigkeit der Knochen Calcium aufzunehmen augenfällig erhöht zu haben scheint. Während bei der D-Hypervitaminose die Mittelwerte der in die Vorder- und Hinterbeinspräparate aufgenommenen Calciummenge 0.20 und 0.24 mg sind, zeigen die Kontrollversuche entsprechende Werte von 0.06 bzw. 0.08 mg auf. Es ist beachtenswert, dass die aufgenommene Calciummenge in der Kontrollserie in keinem einzigen Falle grösser als bei den entsprechenden Ver-

TABELLE 18. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)
D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparates mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 20	1.0 × 3	HRU	500	10.8	8.6	0.22
	(36000 I. E.	HRU	560	10.8	8.7	0.21
	Vitamin D)	FT	900	10.8	7.6	0.32
		FT	930	10.8	8.1	0.27
R 21	1.0 × 3	HRU	550	10.8	7.6	0.32
		HRU	550	10.8	9.1	0.17
		FT	1200	10.8	7.7	0.31
		FT	1220	10.8	7.8	0.3[illegible]
R 22	1.0 × 4	H	490	10.8	9.4	0.14
	(48000 I.E.	RU	330	10.8	9.5	0.13
	Vitamin D)	F	1100	10.8	8.9	0.19
		T	650	10.8	9.8	0.10
R 23	1.0 × 4	F	920	10.8	10.0	0.68
R 24	2.0 × 3	HRU	500	10.8	7.7	0.31
	(72000 I.E.	HRU	610	10.8	9.3	0.15
	Vitamin D)	FT	1050	10.8	8.0	0.28
		FT	1100	10.8	7.5	0.33
R 25	2.0 × 3	HRU	630	10.8	8.9	0.19
		HRU	670	10.8	8.9	0.19
		FT	1010	10.8	8.1	0.27
		FT	1060	10.8	7.6	0.32
R 26	2.0 × 3	HRU	620	10.8	9.0	0.18
		HRU	680	10.8	8.9	0.19
		FT	1090	10.8	8.2	0.26
		FT	1230	10.8	8.9	0.19
R 27	3.0 × 3	HRU	625	10.8	8.6	0.22
	(108000 I.E.	HRU	950	10.8	9.6	0.12
	Vitamin D)	FT	1325	10.8	8.9	0.19
		FT	1350	10.8	9.0	0.18
R 28	3.0 × 3	HRU	785	10.8	7.5	0.33
		HRU	850	10.8	9.7	0.11
		FT	1400	10.8	8.7	0.21
		FT	1775	10.8	8.7	0.21
Durchschnittswert:						
Vorderbeine			616	10.8	8.8	0.20
Hinterbeine			1138	10.8	8.4	0.24

TABELLE 19. *Aufnahme von Calcium in Meerschweinchenknochen.*
(10 ccm Robisons Grundlösung)
Kontrolle.

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
M 15	2.0 × 3	F	1360	14.0	13.2	0.08
		T	1000	14.0	13.4	0.06
		F	1375	14.0	13.2	0.08
		T	1000	14.0	13.1	0.09
M 16	3.0 × 7	F	1030	10.0	9.1	0.09
		T	840	10.0	8.8	0.12
M 17	3.0 × 11	F	850	10.0	9.2	0.08
		T	700	10.0	9.0	0.10
Durchschnittswert: Hinterbeine			**1019**	**12.0**	**11.1**	**0.09**

TABELLE 20. *Aufnahme von Calcium in Meerschweinchenknochen.*
(10 ccm Robisons Grundlösung)
D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg	Aufgenommenes Ca mg
M 18	2.0 × 3	F	1390	14.0	12.2	0.18
	(72000 I.E.	T	990	14.0	12.5	0.15
	Vitamin D)	F	1370	14.0	12.5	0.15
		T	1000	14.0	13.0	0.10
M 19	3.0 × 7	F	930	10.0	8.3	0.17
	(252000 I.E.	T	700	10.0	8.7	0.13
	Vitamin D)					
M 20	3.0 × 11	F	1150	10.0	8.4	0.16
	(396000 I.E.	T	830	10.0	8.2	0.18
	Vitamin D)					
Durchschnittswert: Hinterbeine			**1045**	**12.0**	**10.5**	**0.15**

suchen bei der D-Hypervitaminose war. Der Unterschied erscheint besonders prägnant, wenn man beachtet, dass das Durchschnitts-Gewicht der Präparate in den beiden Serien beinahe gleich war. Die Wirkung des D-Vitamins wird noch von dem Umstande hervorgehoben, dass die aufgenommene Calciummenge der Kontrollserie im grossen ganzen dieselben Werte wie in den Versuchen mit Präparaten von normalen Ratten (Tabellen 1.2 und 3) aufzuweisen hatte. Dieser Sachverhalt deutet ausserdem darauf hin, dass Sesamöl allein keinen nennenswerten Einfluss auf die Aufnahme von Calcium ins Knochengewebe auszuüben scheint.

In der Tabelle 19 und 20 sind die Ergebnisse einer entsprechenden Untersuchung zusammengestellt, in der 16 Hinterbeinspräparate (8 Kontroll) von 6 Meerschweinchen zur Anwendung gekommen sind. Die zugeführte D-Vitaminmenge schwankte zwischen 24000 und 36000 I. E. pro Tag und die Vitaminisierungsdauer war 3—11 Tage. Der Calciumgehalt der Inkubationslösung war 10—14 mg %.

Aus den Versuchen ist zu ersehen, dass auch in diesem Falle die Knochen der D-hypervitaminotischen Tiere konsequent mehr Calcium als die Präparate der entsprechenden Kontrollserie aufgenommen hatten. Die Mittelwerte der zusammengestellten Versuche weisen im ersteren Falle eine aufgenommene Calciummenge von 0.15 gegen 0.09 mg in der Kontrollserie auf.

2. *Aufnahme von organischem Phosphor.*

Für das Studium des vom D-Vitamin auf die Aufnahme von organischem Phosphor ausgeübten Einflusses wurden 40 Knochen-Präparate von 6 Meerschweinchen, von denen die Hälfte als Kontrollmaterial diente, angewandt. Die Vitaminisierung dauerte 3—7 Tage und die Tagesdose schwankte von 12000 bis 36000 I. E. Vitamin D.

Wie aus den Tabellen 21 und 22 zu ersehen ist, hat das D-Vitamin keinen augenfälligen Einfluss auf die Aufnahme von organischem Phosphor in dem geringen Material, das bei diesen Versuchen angewandt wurde, ausgeübt. Die Präparate der Kontrollserie nahmen im Durchschnitt 0.26 mg Phosphor dem Wert von 0.17 mg bei der Hypervitaminose gegenüber auf. Die Resultate der Tabellen 23

TABELLE 21. *Aufnahme von organischem Phosphor in Meerschweinchenknochen.*
(10 ccm Robisons Grundlösung)
Kontrolle.

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initialer TCE-P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 21	2.0 × 3	H	710	14.5	11.5	7.2	0.30
		H	720	14.5	10.9	6.0	0.36
		RU	490	14.5	11.7	8.0	0.28
		RU	480	14.5	13.4	6.5	0.11
Durchschnittswert: Vorderbeine			**600**	**14.5**	**11.9**	**6.9**	**0.26**

TABELLE 22. *Aufnahme von organischem Phosphor in Meerschweinchenknochen.*
(10 ccm Robisons Grundlösung)
D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initialer TCE-P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 22	2.0 × 3	H	720	14.5	14.4	10.3	0.01
	(72000 I.E.	H	730	14.5	14.4	11.9	0.01
	Vitamin D)	RU	500	14.5	11.9	10.3	0.26
		RU	505	14.5	10.5	9.1	0.40
Durchschnittswert: Vorderbeine			**614**	**14.5**	**12.8**	**10.4**	**0.17**

und 24 gehen in der gleichen Richtung. Bei diesen Versuchen nahmen die Vorder- und Hinterbeinspräparate der Kontrollserie eine durchschnittliche Phosphormenge von 0.24 und 0.12 mg und die Präparate der Parallelserie 0.15 bzw. 0.13 mg auf. Eine Beurteilung der Wirkung des D-Vitamins wird von dem Umstande wesentlich erschwert, dass die Aufnahme von Phosphor — im Gegensatz zu dem, wie es mit Calcium der Fall zu sein scheint — eine grosse Veränderlichkeit sogar in Präparaten von denselben Tieren aufzuweisen hatte. Zu irgend einer Schlussfolgerung betreffend den

TABELLE 23. *Aufnahme von organischem Phosphor in Meerschweinchenknochen.* (10 ccm Robisons Grundlösung). *Kontrolle.*

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initialer TCE-P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 23	2.0 × 3	H	900	20.0	17.3	10.0	0.27
		H	900	20.0	17.3	10.6	0.27
		RU	670	20.0	17.1	11.5	0.29
		RU	660	20.0	17.6	11.8	0.24
		F	1410	20.0	17.9	10.9	0.21
		F	1420	20.0	20.0	11.5	0.00
		T	900	20.0	18.4	11.8	0.16
		T	930	20.0	18.1	11.6	0.19
M 24	3.0 × 7	H	1050	20.0	17.0	5.3	0.30
		H	1060	20.0	16.0	5.0	0.40
		RU	750	20.0	18.2	4.8	0.18
		RU	740	20.0	20.0	4.9	0.00
		F	1750	20.0	18.1	5.3	0.19
		F	1730	20.0	17.9	5.7	0.21
		T	1200	20.0	18.6	5.2	0.14
		T	1230	20.0	21.2	5.0	—0.12
Durchschnittswert:							
Vorderbeine			841	20.0	17.6	8.0	0.24
Hinterbeine			1321	20.0	18.8	8.4	0.12

TABELLE 24. *Aufnahme von organischem Phosphor in Meerschweinchenknochen* (10 ccm Robisons Grundlösung). *D-Hypervitaminose.*

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initialer TCE-P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
M 25	2.0 × 3	H	1050	20.0	18.9	9.7	0.11
	(72000 I.E.	H	1060	20.0	19.4	10.3	0.06
	Vitamin D)	RU	730	20.0	17.8	11.5	0.22
		RU	640	20.0	20.0	11.2	0.00
		F	1800	20.0	19.1	10.7	0.09
		F	1800	20.0	18.1	10.7	0.19
		T	1150	20.0	18.6	9.9	0.14
		T	1180	20.0	18.9	10.6	0.11
M 26	3.0 × 7	H	1060	20.0	17.7	4.8	0.23
	(252000 I.E.	H	1070	20.0	18.2	5.3	0.18
	Vitamin D)	RU	780	20.0	17.9	5.1	0.21
		RU	750	20.0	18.1	5.2	0.19
		F	1730	20.0	18.4	5.7	0.16
		F	1750.	20.0	18.6	4.9	0.14
		T	1240	20.0	20.0	5.3	0.00
		T	1190	20.0	17.7	5.6	0.23
Durchschnittswert:							
Vorderbeine			893	20.0	18.5	7.9	0.15
Hinterbeine			1480	20.0	18.7	7.9	0.13

Einfluss von Vitamin D auf die finale anorganische Phosphor-Fraktion dürfte man auch kaum kommen können, da auch die letztere in den verschiedenen Versuchen der beiden Serien zwischen bedeutenden Grenzen schwankte.

3. *Aufnahme von Calcium und organischem Phosphor.*

Die obigen Beobachtungen gaben Veranlassung zu Versuchen, welche das Studium der Einwirkung von D-Vitamin auf die Aufnahme von Calcium und organischem Phosphor beim gleichzeitigen Anwesen dieser Stoffe in der Inkubationslösung zum Ziel hatten. Das Material umfasste 24 Präparate von 4 Kontroll- und 3 hypervitaminotischen Ratten. Die letzterwähnten Tiere erhielten 24000 I. E. D-Vitamin täglich während 4 Tagen.

TABELLE 25. *Aufnahme von Calcium und organischem Phosphor in Rattenknochen.*

(10 ccm Robisons Grundlösung)

Kontrolle.

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg	Finales Ca mg	Aufgenommenes Ca mg	Initialer TCE-P mg	Finaler TCE-P mg	Finaler anorg. P mg	Aufgenommener P mg
R 29	2.0 × 4	H	500	6.5	6.3	0.02	10.0	7.3	5.7	0.27
		RU	300	6.5	6.5	0.00	10.0	7.9	5.5	0.21
		F	1060	6.5	5.6	0.09	10.0	7.0	6.3	0.30
		T	700	6.5	5.8	0.07	10.0	7.9	6.0	0.21
R 30	2.0 × 4	H	270	9.0	9.0	0.00	8.0	6.7	2.8	0.13
		RU	200	9.0	8.9	0.01	8.0	5.3	3.5	0.27
		F	500	9.0	8.6	0.04	8.0	5.7	5.4	0.23
		T	390	9.0	8.4	0.06	8.0	8.0	7.7	0.00
R 31	2.0 × 4	H	400	9.0	8.1	0.09	8.0	7.3	6.6	0.07
		RU	270	9.0	8.1	0.09	8.0	7.5	7.1	0.05
R 32	2.0 × 4	F	750	9.0	8.5	0.05	8.0	8.7	5.8	−0.07
		T	500	9.0	8.2	0.08	8.0	6.4	6.0	0.16
Durchschnittswert:										
Vorderbeine			323	8.2	7.8	0.04	8.7	7.0	5.2	0.17
Hinterbeine			650	8.2	7.5	0.07	8.7	7.3	6.2	0.14

TABELLE 26. *Aufnahme von Calcium und organischem Phosphor in Rattenknochen.*

(10 ccm Robisons Grundlösung)

D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg	Initialer TCE-P mg %	Finaler TCE-P mg	Finaler anorg. P mg %	Aufgenommener P mg
R 33	2.0 × 4	H	450	6.5	5.1	0.14	10.0	6.7	4.5	0.33
	(96000 I.E.	RU	300	6.5	5.2	0.13	10.0	6.7	4.1	0.33
	Vitamin D)	F	950	6.5	5.6	0.09	10.0	7.6	4.3	0.24
		T	670	6.5	5.7	0.08	10.0	7.8	4.8	0.22
R 34	2.0 × 4	H	370	9.0	7.5	0.15	8.0	6.1	5.8	0.19
		RU	260	9.0	7.8	0.12	8.0	6.1	5.8	0.19
		F	760	9.0	7.4	0.16	8.0	5.5	5.2	0.25
		T	560	9.0	7.4	0.16	8.0	5.3	5.1	0.27
R 35	2.0 × 4	H	400	9.0	7.1	0.19	8.0	6.1	5.9	0.19
		RU	280	9.0	6.8	0.22	8.0	6.1	5.9	0.19
		F	1000	9.0	7.6	0.14	8.0	6.3	6.1	0.17
		T	650	9.0	7.7	0.13	8.0	6.3	6.5	0.17
Durchschnittswert:										
Vorderbeine			343	8.2	6.6	0.16	8.7	6.3	5.3	0.24
Hinterbeine			762	8.2	6.9	0.13	8.7	6.5	5.3	0.22

Die Tabellen 25 und 26 bestätigen weiterhin die früher gemachte Wahrnehmung, dass das D-Vitamin die Knochen mehr »Calciumhungrig» als unter normalen Verhältnissen macht. Während die Vorderbeinspräparate der Kontrollserie durchschnittlich 0.04 mg Calcium und die Hinterbeinspräparate 0.07 mg aufgenommen hatten, waren die entsprechenden Werte bei der Hypervitaminose 0.16 und 0.13 mg Calcium. Ein ebenso deutlicher Effekt des D-Vitamins, was die Aufnahme von organischem Phosphor betrifft, konnte dagegen nicht wahrgenommen werden. Es scheint hierbei eine Tendenz zu einer grösseren Aufnahme bei der D-Hypervitaminose dennoch vorzuliegen, da in dieser Serie die Vorder- und Hinterbeinspräparate durchschnittlich 0.24 und 0.22 mg Phosphor aufgenommen hatten, wogegen die entsprechenden Werte der Kontroll-Versuche 0.17 bzw. 0.14 mg Phosphor waren. Es ist jedoch wahr-

scheinlich noch zu früh aus diesem Versuche irgend welche Schlüsse über die Einwirkung des Vitamin D auf die Aufnahme von Phosphor zu ziehen. Was die Menge der finalen anorganischen Phosphorfraktion betrifft, so deutet auch dieser Versuch darauf hin, dass dieselbe vom Vitamin D nicht beeinflusst werden dürfte.

4. *Retention von Calcium.*

Im Kenntnis des Zusammenhanges, welcher zwischen der Aufnahme und der Retention von Calcium im Knochengewebe zu bestehen scheint, erschien das Studium des vom Vitamin D auf den letzterwähnten Mechanismus ausgeübten Einflusses Interesse zu erbieten. Zu diesem Zwecke wurden insgesamt 30 Vorder- und Hinterbeinspräparate von 5 Kontroll- und 5 D-hypervitamino-

TABELLE 27. *Retention von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)
Kontrolle.

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg	Abgegebenes Ca mg
R 15	1.0 × 4	H	590	0	0.34
		F	1250	0	0.38
		T	870	0	0.37
R 36	1.0 × 4	HRU	650	0	0.55
		FT	1300	0	0.67
		FT	1300	0	0.54
R 37	1.0 × 4	HRU	540	0	0.49
		HRU	570	0	0.52
		FT	1100	0	0.65
		FT	1140	0	0.63
R 38	1.0 × 4	HRU	555	0	0.47
		FT	1090	0	0.56
R 39	1.0 × 4	HRU	620	0	0.42
		FT	1300	0	0.58
Durchschnittswert:					
Vorderbeine			588	0	0.47
Hinterbeine			1169	0	0.55

TABELLE 28. *Retention von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)
D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initiales Ca mg	Abgegebenes Ca mg
R 27	1.0 × 4	HRU	650	0	0.47
	(48000 I.E.)	HRU	830	0	0.40
		FT	1370	0	0.58
R 28	1.0 × 4	HRU	700	0	0.44
		HRU	720	0	0.44
		FT	1650	0	0.50
R 40	1.0 × 4	HRU	600	0	0.40
		FT	1155	0	0.59
		FT	1205	0	0.44
R 41	1.0 × 4	H	520	0	0.38
		RU	350	0	0.36
		F	1200	0	0.36
		T	730	0	0.35
R 42	1.0 × 4	H	500	0	0.31
		F	1070	0	0.33
		T	750	0	0.32
Durchschnittswert:					
Vorderbeine			609	0	0.40
Hinterbeine			1141	0	0.43

tischen Ratten angewandt. Die Vitaminisierung geschah bei diesem Versuche vermittelst einer täglichen Zuführung von 12000 I. E. Vitamin D im Laufe von 4 Tagen.

Der Versuch wies bei den Knochen der D-hypervitaminotischen Ratten eine Tendenz auf, Calcium besser als die Präparate der Kontrollserie zu fixieren (Tabelle 27, 28). Im ersteren Falle gaben die Vorder- und Hinterbeinspräparate im Durchschnitt 0.40 und 0.43 mg Calcium an die Natriumchloridlösung ab, wogegen die entsprechenden Zahlen der Kontrollserie 0.47 und 0.55 mg waren. Da, wie oben erwähnt, das D-Vitamin einen stimulierenden Einfluss auf die Aufnahme von Calcium zu haben scheint, dürfte hierdurch der »Calciumhunger» des Knochengewebes bei der D-Hyper-Vitaminose s. z. s. im doppelten Sinne hervorgehoben.

5. *Retention von Phosphor.*

Für entsprechende, die Einwirkung des Vitamins D auf die Retention von Phosphor beleuchtende Versuche sind insgesamt 37 Präparate von 5 Kontroll- und 5 D-hypervitaminotischen Ratten zur Anwendung gekommen. Die Zuführung von Vitamin D fand während 4 Tagen in Tagesdosen von 24000 I. E. statt.

Die Tabellen 29 und 30 zeigen, dass kein nachweisbarer Unterschied in der Menge der abgegebenen totalen säurelöslichen Phosphorfraktion in den Parallelserien verspürt werden kann. Die

TABELLE 29. *Retention von Phosphor in Rattenknochen.*
(10 ccm Robisons Grundlösung)
Kontrolle.

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initialer P mg	Abgegebener TCE-P mg	Abgegebener anorg. P mg
R 29	2.0 × 4	H	500	0	0.40	0.23
		RU	350	0	0.37	0.20
		F	1060	0	0.37	0.26
		T	770	0	0.40	0.25
R 30	2.0 × 4	H	270	0	0.16	0.12
		RU	200	0	0.12	0.12
		F	500	0	0.16	0.16
		T	400	0	0.15	0.15
R 31	2.0 × 4	H	390	0	0.24	0.23
		RU	250	0	0.26	0.19
		F	900	0	0.32	0.32
		T	600	0	0.40	0.26
R 32	2.0 × 4	H	330	0	0.39	0.32
		RU	200	0	0.21	0.20
		F	760	0	0.23	0.22
		T	500	0	0.37	0.19
R 43	2.0 × 4	H	360	0	0.82	0.51
		F	820	0	0.74	0.42
		T	520	0	0.74	0.42
Durchschnittswert:						
Vorderbeine			317	0	0.33	0.24
Hinterbeine			683	0	0.39	0.27

TABELLE 30. *Retention von Calcium in Rattenknochen.*
(10 ccm Robisons Grundlösung)
D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initialer P mg	Abgegebener TCE-P mg	Abgegebener anorg. P mg
R 33	2.0 × 4	H	450	0	0.40	0.24
	(96000 I.E.	RU	300	0	0.41	0.18
	Vitamin D)	F	950	0	0.47	0.07
		T	600	0	0.47	0.22
R 34	2.0 × 4	H	360	0	0.18	0.65
		T	540	0	0.10	0.66
R 35	2.0 × 4	H	400	0	0.22	0.06
		RU	280	0	0.20	0.06
		F	820	0	0.20	0.04
		T	600	0	0.12	0.12
R 44	2.0 × 4	H	480	0	0.47	0.25
		RU	330	0	0.78	0.19
		F	1100	0	0.52	0.20
		T	710	0	0.52	0.29
R 45	2.0 × 4	H	370	0	0.21	0.21
		RU	270	0	0.13	0.07
		F	780	0	0.17	0.17
		T	550	0	0.17	0.14
Durchschnittswert:						
Vorderbeine			360	0	0.33	0.15
Hinterbeine			739	0	0.30	0.15

Vorder- und Hinterbeinspräparate der Kontrolltiere gaben durchschnittlich 0.33 und 0.39 mg Phosphor ab, wogegen die entsprechenden Werte für die hypervitaminotischen 0.33 bzw. 0.30 mg waren. Dagegen hat die aus den Knochen ausgewanderte anorganische Phosphorfraktion eine relative Abnahme bei der D-Hypervitaminose (0.15 bzw. 0.15 mg) den Versuchen der Kontrollserie gegenüber (0.24 bzw. 0.27 mg) aufzuweisen. Die beiden abgegebenen Phosphorfraktionen scheinen jedoch in den Versuchen eine ziemlich grosse Veränderlichkeit aufzuweisen, weshalb es voreilig wäre, irgendwelche unmittelbare Schlüsse über die Einwirkung des D-Vitamins auf die Fixierung von Phosphor in den Präparaten zu ziehen.

TABELLE 31. *Retention von Calcium und Phosphor in Meerschweinchenknochen.* (10 ccm Robisons Grundlösung)
Kontrolle.

Nr.	Verabreichte Menge Sesamöl ccm	Knochen	Gewicht des Präparats mg	Initiale Ca und P mg	Abgegebenes Ca mg	Abgegebener TCE-P mg	Abgegebener anorg. P mg
M 16	3.0 × 7	F	1060	0	0.32	0.17	0.07
		T	800	0	0.29	0.20	—
M 17	3.0 × 11	F	900	0	0.31	0.34	0.17
		T	680	0	0.27	0.32	0.20
Durchschnittswert: Hinterbeine			860	0	0.30	0.26	0.15

TABELLE 32. *Retention von Calcium und Phosphor in Meerschweinchenknochen.* (10 ccm Robinsons Grundlösung)
D-Hypervitaminose.

Nr.	Verabreichte Menge Vigantol ccm	Knochen	Gewicht des Präparats mg	Initiale Ca und P mg	Abgegebenes Ca mg	Abgegebener TCE-P mg	Abgegebener anorg. P mg
M 27	3.0 × 5	F	1000	0	0.20	0.28	0.11
	(180000 I.E. Vitamin D)	T	730	0	0.23	0.22	0.05
M 19	3.0 × 7	F	950	0	0.23	0.29	0.13
	(252000 I.E. Vitamin D)	T	680	0	0.19	0.20	0.07
M 20	3.0 × 11	F	1160	0	0.25	0.34	0.22
	(396000 I.E. Vitamin D)	T	850	0	0.22	0.27	0.15
Durchschnittswert: Hinterbeine			895	0	0.22	0.27	0.12

6. *Retention von Calcium und Phosphor.*

Zu Kontrollzwecken wurde eine kurze Versuchsserie vorgenommen, in der die Retention von Calcium und Phosphor in 10 Hinterbeinspräparaten von 5 Meerschweinchen, von denen 2 Kontrolltiere waren, gleichzeitig beobachtet wurde. Die Vitamini-

sierung wurde durch eine tägliche Zuführung von 36000 I. E. D-Vitamin im Laufe von 5—11 Tagen bewirkt.

Die Ergebnisse der Tabelle 31 und 32 scheinen die frühere Wahrnehmung zu bestätigen, dass bei D-Hypervitaminose die Knochen eine Tendenz aufweisen Calcium besser als bei den Kontrollversuchen fixieren zu können. Im ersteren Falle gaben die Präparate an die Basallösung eine durchschnittliche Calciummenge von 0.22 mg einem Werte von 0.30 mg in der Kontrollserie gegenüber ab. Obwohl dieser Unterschied nicht besonders auffallend ist, zeigen jedoch die Versuche, dass die Knochen bei D-Hypervitaminose in keinem Falle mehr Calcium als in den Kontrollversuchen abgegeben hatten.

Was den ausdiffundierten totalen säurelöslichen Phosphor betrifft, so kann kein nennenswerter Unterschied zwischen den Versuchen der Kontroll- und der D-Hypervitaminoseserie (0.26 bzw. 0.27 mg) verspürt werden, und dieselbe Beobachtung scheint auch für die entsprechende anorganische Phosphorfraktion gültig zu sein (0.15 bzw. 0.12 mg). Die Veränderlichkeit der abgegebenen Phosphormenge kam auch bei diesen Versuchen zum Vorschein.

7. *Serumcalcium und Blutphosphor bei D-Hypervitaminose.*

Im Anschluss an die obenerwähnten Versuche wurde der Kontrolle wegen eine Serie von Calcium- und Phosphorbestimmungen im Blute einiger Versuchstiere ausgeführt. Zu diesem Zwecke wurde das Blut von 31 Meerschweinchen, von denen 26 bei den früher erwähnten Versuchen zur Anwendung gekommen waren, entnommen. Der Calciumgehalt wurde in dem Serum, die Phosphorfraktionen im Vollblute bestimmt.

Aus der Tabelle 33 ist zu ersehen, dass der Calciumgehalt im Serum der D-hypervitaminoschen Tiere durchschnittlich etwas höher war als in den Serien, bei welchen das Serum normaler und Kontroll-Tiere zur Anwendung kam. Das D-Vitamin erwies sich ebenfalls einen erhöhenden Einfluss sowohl auf die totale säurelösliche wie auch auf die anorganische Phosphorfraktion auszuüben. Diese Ergebnisse bestätigen somit die im Zusammenhange mit der Literaturübersicht erwähnten, über diese Seite des Wirkungsmechanismus des Vitamins D gemachten Beobachtungen.

TABELLE 33. *Serumcalcium und Blutphosphor bei Meerschweinchen unter verschiedenen Bedingungen.*

A. Normale Tiere

Nr.	Ca mg %	TCE-P mg %	anorg. P mg %
M 1	11.7	21.1	3.2
M 2	11.1	24.3	7.9
M 3	11.0	28.3	3.9
M 5	12.3	23.5	3.1
M 6	11.2	32.1	—
M 7	10.3	23.2	7.4
M 8	11.5	23.0	4.6
M 9	11.6	23.0	5.9
M 10	10.5	32.5	8.5
M 11	10.5	34.7	5.1
M 12	11.2	30.3	3.9
M 13	10.7	27.1	5.8
M 14	11.0	24.6	6.6
Durchschnittswert:	**11.1**	**26.7**	**5.5**

B. Kontrolltiere

Nr.	Verabreichte Menge Sesamöl ccm	Ca mg %	TCE-P mg %	anorg. P mg %
M 15	2.0 × 3	13.1	23.9	5.7
M 21	»	10.1	21.8	6.7
M 23	»	10.6	26.3	4.8
M 28	3.0 × 5	10.3	29.5	5.8
M 29	»	10.0	20.9	4.3
M 16	3.0 × 7	10.6	26.9	5.2
M 24	»	10.0	18.1	4.9
M 17	3.0 × 11	11.9	32.4	4.9
Durchschnittswert:		**10.8**	**24.9**	**5.3**

C. D-hypervitaminotische Tiere

Nr.	Verabreichte Menge Vigantol ccm	Ca mg %	TCE-P mg %	anorg. P mg %
M 18	2.0 × 3	13.8	21.8	6.2
M 22	»	12.1	28.2	6.2
M 25	»	11.9	27.4	6.0
M 27	3.0 × 5	11.7	29.5	5.8
M 30	»	10.6	31.2	10.0
M 31	»	11.1	33.1	7.6
M 32	»	13.6	41.4	8.3
M 26	3.0 × 7	14.0	33.9	10.3
M 19	»	12.4	26.9	7.3
M 20	3.0 × 11	11.7	39.1	9.3
Durchschnittswert:		**12.3**	**31.2**	**7.7**

G. Aufnahme und Retention von Calcium und Phosphor im Knochengewebe bei D-Avitaminose.

Um eine so vollständige Vorstellung wie möglich von der vom D-Vitamin beim Calcium- und Phosphormetabolismus im Knochen-Gewebe gespielten Rolle im Ramen dieser in vitro ausgeführten Versuche zu erhalten, ist in den nachstehend beschriebenen Versuchen der entsprechende Stoffwechsel — ausgehend von den bei der D-Hypervitaminose gemachten Beobachtungen — bei der D-Avitaminose studiert worden. Bei diesen letzteren Versuchen wurden die Versuchstiere mit der rachitogenen Kost Mc Collums aufgefüttert. Auch bei diesen Versuchen geschah die Auswahl der Tiere auf eine grossmöglichste Gleichförmigkeit der Präparate in den Parallel erien hin. Es kamen insgesamt 133 Knochenpräparate zur Anwendung, davon 65 als Kontrollmaterial. Die Versuchstiere waren 22 Ratten (10 Kontrolltiere) und 15 Kaninchen (7 Kontrolltiere). Da die Knochenpräparate der jungen Ratten bei diesen Versuchen in den meisten Fällen viel kleiner als in den früher besprochenen Experimenten waren, schien es als berechtigt die Menge der Inkubationslösungen in einigen Serien auf 4—5 ccm zu vermindern, um den Calcium- und Phosphormetabolismus in diesen Präparaten deutlicher zum Vorschein kommen zu lassen. Bei den einzelnen Versuchen ist das Inkubationsmilieu für die Präparate der Kontroll- und der Rachitistiere genau das gleiche gewesen.

1. *Aufnahme von Calcium.*

In der Tabelle 34 und 35 sind die Ergebnisse der die Aufnahme von Calcium in 14 Hinterbeinspräparaten von 4 Kontroll- und 4 rachitischen Ratten beleuchtenden Parallelversuche zusammengestellt. Der Calciumgehalt der Inkubationslösung war in diesen Versuchen 12.2 mg %.

Aus diesen Versuchen geht hervor, dass die Gewichtszunahme der Ratten in den beiden Gruppen im grossen ganzen verhältnismässig befreidigend gewesen ist. Hinsichtlich der Aufnahme von Calcium wurde die beachtenswerte Wahrnehmung gemacht, dass die Knochenpräparate in der Rachitisserie diesen Stoff nicht ver-

TABELLE 34. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)
Kontrolle.

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 46	12	41	41	FT	460	12.2	10.4	0.18
R 47	58	46	70	FT	700	12.2	12.3	—0.01
				FT	750	12.2	12.0	0.02
R 48	58	41	90	FT	770	12.2	12.7	—0.05
				FT	750	12.2	12.2	0.00
R 49	60	39	40	FT	610	12.2	13.6	—0.14
				FT	640	12.2	13.2	—0.10
Durchschnittswert: Hinterbeine					**669**	**12.2**	**12.3**	**—0.01**

TABELLE 35. *Aufnahme von Calcium in Rattenknochen.*
(10 ccm physiologische Natriumchloridlösung)
D-Avitaminose.

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
R 50	50	40	60	FT	720	12.2	15.1	—0.29
				FT	720	12.2	14.5	—0.23
R 51	50	37	55	FT	560	12.2	15.7	—0.35
				FT	600	12.2	15.1	—0.29
R 52	51	42	61	FT	620	12.2	13.5	—0.13
R 53	60	43	77	FT	690	12.2	17.5	—0.53
				FT	690	12.2	13.2	—0.10
Durchschnittswert: Hinterbeine					**657**	**12.2**	**14.9**	**—0.27**

werten zu können schienen. In allen Versuchen dieser Serie hat anstatt einer Aufnahme eine nachweisbare Abgabe von Calcium aus den Präparaten an die Inkubationslösung stattgefunden. Der Mittelwert der ausdiffundierten Calciummenge war 0.27 mg. Hier scheint also das Verhältnis genau umgekehrt zu sein wie es bei der

TABELLE 36. *Aufnahme von Calcium in Kaninchenknochen.*
(10 ccm physiologische Natriumchloridlösung)
Kontrolle.

Nr.	Diätdauer Tage	Anfangs-Gewicht der Kaninchen g	Endgewicht der Kaninchen g	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
K 1	18	345	320	F	2150	12.2	11.6	0.06
				F	2150	12.2	10.9	0.13
K 2	24	450	450	F	3000	12.2	9.0	0.32
				F	3000	12.2	8.7	0.35
				T	2400	12.2	8.6	0.36
				T	2300	12.2	10.2	0.20
K 3	30	300	210	F	1750	12.2	10.7	0.15
				F	1700	12.2	10.4	0.18
				T	1420	12.2	11.4	0.08
				T	1520	12.2	11.8	0.04
K 4	57	520	480	F	3480	12.2	11.4	0.08
				T	2870	12.2	12.4	—0.02
Durchschnittswert: Hinterbeine					**2312**	**12.2**	**10.6**	**0.16**

TABELLE 37. *Aufnahme von Calcium in Kaninchenknochen.*
(10 ccm physiologische Natriumchloridlösung)
D-Avitaminose.

Nr.	Diätdauer Tage	Anfangsgewicht der Kaninchen g	Endgewicht der Kaninchen g	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Aufgenommenes Ca mg
K 5	9	435	280	T	1900	12.2	13.1	—0.09
				T	1900	12.2	12.1	0.01
K 6	24	395	300	T	1720	12.2	11.6	0.06
				T	1720	12.2	11.2	0.10
K 7	30	360	320	F	2100	12.2	11.6	0.06
				F	2200	12.2	11.1	0.11
				T	1750	12.2	12.5	—0.03
				T	1750	12.2	13.2	—0.10
K 8	30	320	300	T	1800	12.2	10.4	0.18
K 9	30	290	270	T	1620	12.2	10.9	0.13
K 10	30	330	340	F	2320	12.2	10.8	0.14
				F	2320	12.2	10.4	0.18
				T	2050	12.2	11.1	0.11
				T	1850	12.2	12.1	0.01
Durchschnittswert: Hinterbeine					**1929**	**12.2**	**11.6**	**0.06**

Hypervitaminose war. Auch in der Kontrollserie zeigten die Präparate eine verminderte Calciumaufnahmefähigkeit, obwohl diese »Minderwertigkeit» hier nicht so auffallend war wie in der Rachitisserie. Der Mittelwert der finalen Calciummenge zeigt jedoch, dass auch hier die Präparate in den meisten Versuchen Calcium abgegeben haben (0.01 mg).

Die Tabellen 36 und 37 veranschaulichen eine entsprechende Versuchsserie, in der 26 Hinterbeinspräparate von 4 Kontroll- und 6 D-avitaminotischen Kaninchen zur Anwendung kamen. Der Calciumgehalt der Inkubationslösung war auch bei diesen Versuchen 10.2 mg %.

Bei der Beschreibung der Arbeitsmethodik ist hervorgehoben worden, dass Kaninchen die rachitogene Kost Mc Collums sich ungern zunutze machten. Die Tiere assen schlecht und viele von ihnen wiesen schon einige Tage nach dem Beginne der Diät Zeichen von Inanition auf. Dieser Umstand kam u. a. zum Ausdruck durch eine Abnahme im Gewicht der Tiere in beinahe allen Versuchen der beiden Serien. Was die Aufnahme von Calcium betrifft, so geht aus den Versuchen hervor, dass eine Adsorption in den meisten Fällen der beiden Gruppen statt gefunden ist, wobei jedoch die Präparate der »Rachitisserie» eine Neigung aufwiesen weniger Calcium als die Kontrollpräparate aufzunehmen. Während der Mittelwert der aufgenommenen Calciummenge im ersteren Falle 0.06 mg war, hatte die Kontrollserie einen solchen von 0.16 aufzuweisen. In den beiden Gruppen schien die Fähigkeit der Knochen Calcium zu verwerten ziemlich grossen Schwankungen unterworfen zu sein. In der Kontrollserie machte sich bei den Präparaten eine Tendenz weniger Calcium bei verlängerter Diätdauer aufzunehmen fühlbar.

2. *Aufnahme von organischem Phosphor.*

Als Versuchsmaterial für Untersuchungen über die Aufnahme von organischem Phosphor im Knochengewebe bei der D-Avitaminose kamen 34 Vorder- und Hinterbeinspräparate von 9 Ratten, darunter 4 Kontrolltieren, zur Anwendung. Bei diesen Versuchen war die Phosphorkonzentration der Inkubationslösung 20 mg % (Die absolute Menge: 0.8 mg Phosphor).

TABELLE 38. *Aufnahme von organischem Phosphor in Rattenknochen.*
(4 ccm Robisons Grundlösung)
Kontrolle.

Nr.	Diätdauer Tage	Anfangs-gewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initialer TCE-P mg %	Finaler TCE-P mg %	Finaler anorg. P mg %	Aufgenommener P mg
R 54	17	30	35	HRU	200	20.0	18.3	7.5	0.07
				HRU	210	20.0	18.6	7.3	0.06
				F	170	20.0	20.0	6.6	0.00
				F	180	20.0	20.0	6.4	0.00
				T	160	20.0	17.3	6.1	0.11
				T	150	20.0	18.9	5.9	0.04
R 55	24	39	40	HRU	220	20.0	18.9	6.8	0.04
				HRU	210	20.0	19.7	5.7	0.01
				F	220	20.0	19.1	6.1	0.04
				F	215	20.0	19.7	6.2	0.01
				T	180	20.0	21.0	5.5	—0.04
				T	195	20.0	20.0	5.2	0.00
R 56	39	39	81	HRU	250	20.0	18.2	10.6	0.07
				F	300	20.0	17.3	9.7	0.11
				T	250	20.0	18.8	9.1	0.05
R 57	41	53	95	H	200	20.0	15.5	8.2	0.18
				RU	160	20.0	14.4	9.2	0.22
				T	250	20.0	15.5	8.7	0.18
Durchschnittswert:									
Vorderbeine					207	20.0	17.7	7.9	0.09
Hinterbeine					206	20.0	18.9	6.9	0.04

Die Tabellen 38 und 39 zeigen, dass irgend welcher augenscheinlicher Unterschied in der Aufnahme von Phosphor in den beiden Gruppen sich nicht entdecken lässt. Die Vorder- und Hinterbeinspräparate der Kontrollserie hatten im Durchschnitt eine Phosphormenge von 0.09 bzw. 0.04 mg aufgenommen, während die entsprechenden Zahlen der Rachitisserie 0,06 und 0.06 mg waren. In den beiden Serien schwankte die Aufnahme von Phosphor in den einzelnen Versuchen zwischen ziemlich weiten Grenzen. Hinsichtlich der finalen anorganischen Phosphorfraktion der

TABELLE 39. *Aufnahme von organischem Phosphor in Rattenknochen.*
(4 ccm Robisons Grundlösung)
D-Avitaminose.

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initialer TCE-P mg %	Finaler TCE-P mg %	Finaler anorg. P mg "	Aufgenommener P mg %
R 58	20	34	37	HRU	205	20.0	16.7	7.8	0.13
				HRU	210	20.0	16.9	7.3	0.12
				F	190	20.0	17.7	6.4	0.09
				F	200	20.0	19.3	7.7	0.03
				T	160	20.0	18.9	5.9	0.04
				T	150	20.0	19.2	5.6	0.03
R 59	21	32	37	HRU	200	20.0	18.8	8.0	0.05
				HRU	210	20.0	20.0	7.7	0.00
				F	190	20.0	19.4	6.5	0.02
				F	190	20.0	18.2	6.5	0.07
				T	195	20.0	17.6	6.0	0.10
				T	185	20.0	16.0	5.5	0.16
R 60	25	42	50	HRU	220	20.0	19.7	7.0	0.01
				F	225	20.0	20.3	6.4	—0.01
				T	210	20.0	19.1	4.8	0.04
				T	200	20.0	22.1	5.7	—0.08
R 61	38	40	48	HRU	225	20.0	18.8	8.5	0.05
				F	240	20.0	19.4	8.8	0.02
				T	230	20.0	19.1	9.1	0.04
R 62	41	30	57	F	220	20.0	15.5	9.2	0.18
				T	210	20.0	16.0	9.2	0.16
Durchschnittswert:									
Vorderbeine					212	20.0	18.5	7.7	0.06
Hinterbein					200	20.0	18.5	6.9	0.06

Inkubationslösung konnte auch hier kein nennenswerter Unterschied zwischen den Parallelserien verspürt werden. Bei den Versuchen mit Vorderbeinspräparaten war in den Kontroll- und Rachitisserien die Konzentration der finalen anorganischen Phosphorfraktion in der Inkubationslösung 7.9 und 7.7 mg %, wogegen die entsprechenden Werte in den beiden Serien mit Hinterbeins-Präparaten gleich 6.9 war. Die Ergebnisse gehen somit in derselben

Tabelle 40. *Retention von Calcium in Rattenknochen.*
(5 ccm physiologische Natriumchloridlösung)
Kontrolle.

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Abgegebenes Ca mg
R 46	12	41	41	HRU	300	0	4.4	0.22
R 63	41	53	90	HRU	420	0	6.0	0.30
				HRU	400	0	6.6	0.33
R 47	58	46	70	HRU	380	0	4.2	0.21
				HRU	420	0	3.4	0.17
R 48	58	41	90	HRU	500	0	4.7	0.24
R 49	60	39	49	HRU	380	0	4.4	0.22
Durchschnittswert: Vorderbeine					**400**	**0**	**4.8**	**0.24**

TABELLE 41. *Retention von Calcium in Rattenknochen.*
(5 ccm physiologische Natriumchloridlösung)
D-Avitaminose.

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initiales Ca mg %	Finales Ca mg %	Abgegebenes Ca mg
R 64	41	65	150	HRU	410	0	6.8	0.34
				HRU	350	0	5.7	0.29
R 50	50	40	60	HRU	300	0	6.5	0.33
				HRU	350	0	5.5	0.28
R 51	50	37	55	HRU	350	0	5.5	0.28
				HRU	350	0	6.2	0.31
R 52	51	42	61	HRU	360	0	5.6	0.28
R 65	52	43	77	HRU	320	0	9.7	0.49
Durchschnittswert: Vorderbiene					**349**	**0**	**6.4**	**0.32**

Richtung wie in den Versuchen bei D-Hypervitaminose, bei welcher kein unmittelbarer Einfluss des D-Vitamins auf die Aufnahme von Phosphor beobachtet werden konnte.

TABELLE 42. *Retention von Phosphor in Rattenknochen.* (4 ccm Robisons Grundlösung). *Kontrolle.*

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initialer P mg	Abgegebener TCE-P mg	Abgegebener anorg. P mg
R 66	9	33	39	H	130	0	0.19	0.09
				H	120	0	0.18	0.05
				RU	110	0	0.09	0.07
				RU	105	0	0.07	0.05
				F	205	0	0.22	0.04
				F	215	0	0.18	0.10
				T	200	0	0.16	0.08
				T	190	0	0.14	0.04
R 56	39	39	81	HRU	255	0	0.16	0.16
				F	300	0	0.13	0.13
				T	245	0	0.12	0.12
R 57	41	53	95	H	180	0	0.18	0.18
				RU	170	0	0.15	0.13
				F	330	0	0.30	0.24
Durchschnittswert:								
Vorderbeine					**153**	**0**	**0.15**	**0.10**
Hinterbeine					**241**	**0**	**0.18**	**0.11**

TABELLE 43. *Retention von Phosphor in Rattenknochen.* (4 ccm Robisons Grundlösung). *D-Avitaminose.*

Nr.	Diätdauer Tage	Anfangsgewicht der Ratten g	Endgewicht der Ratten g	Knochen	Gewicht des Präparats mg	Initialer P mg	Abgegebener TCE-P mg	Abgegebener anorg. P mg
R 67	7	40	30	H	115	0	0.14	0.08
				H	115	0	0.13	0.08
				RU	100	0	0.09	0.06
				RU	105	0	0.08	0.07
				F	200	0	0.13	0.11
				F	190	0	0.13	0.09
				T	180	0	0.18	0.08
				T	170	0	0.13	0.07
R 61	38	40	48	HRU	220	0	0.15	0.15
				F	240	0	0.15	0.14
				T	225	0	0.14	0.13
R 62	41	30	57	HRU	250	0	0.18	0.18
				F	230	0	0.17	0.17
				T	210	0	0.16	0.16
Durchschnittswert:								
Vorderbeine					**151**	**0**	**0.13**	**0.10**
Hinterbeine					**206**	**0**	**0.15**	**0.12**

3. *Retention von Calcium.*

Die Tabellen 40 und 41 veranschaulichen die Abgabe von Calcium an eine physiologische Natriumchloridlösung aus 15 Vorderbeinspräparaten von 5 Kontroll- und 5 rachitischen Ratten. Einige dieser Ratten sind als Versuchstiere in den die Aufnahme von Calcium in die Hinterbeinspräparate bei Rachitis beleuchtenden Parallelserien angewandt worden.

Wie es aus den Tabellen 40 und 41 hervorgeht, zeigen die rachitischen Knochen eine Tendenz Calcium schwächer als die Kontroll-Präparate fixieren zu können. In dem ersteren Falle gaben die Präparate durchschnittlich 0.32 mg Calcium ab und 0.24 mg in der Kontrollserie. Bei Rachitis dürfte also das Verhältnis dem bei der D-Hypervitaminose herrschenden ein gerade entgegengesetztes sein.

4. *Retention von Phosphor.*

Für Untersuchungen betreffend die Retention von Phosphor sind 28 Knochenpräparate von 6 Ratten, von denen die Hälfte Kontrolltiere waren, zur Anwendung gekommen.

Die Ergebnisse sind in der Tabelle 42 und 43 zusammengestellt worden. Aus den Versuchen ist zu ersehen, dass irgendwelcher nennenswerter Unterschied zwischen den in den beiden Gruppen aus den Präparaten herausgewanderten Phosphormengen nicht beobachtet werden kann. Während die Vorder- und Hinterbeins-Präparate der Kontrollserie im Durchschnitt eine totale säurelösliche Phosphormenge von 0.15 und 0.18 mg abgaben, waren die entsprechen Zahlen in der Rachitisserie 0.13 bzw. 0.15 mg. In den meisten Fällen lag der herausdiffundierte Phosphor hauptsächlich in anorganischer Form vor, jedoch konnte kein auffallender Unterschied in der Grösse dieser Fraktion in den Parallelversuchen entdeckt werden.

Für Kontrollzwecke wurde ausserdem die Abgabe von Phosphor in einem Parallelversuche beobachtet, für welchen insgesamt 25 Vorderbeinspräparate von 5 Kontroll- und 6 D-avitaminotischen Kaninchen angewandt wurden. Da die anorganische Phosphor-Sälze die hauptsächlichsten Phosphorverbindungen im Knochen-

TABELLE 44. *Retention von anorganischem Phosphor in Kaninchenknochen.* (10 ccm physiologische Natriumchloridlösung) *Kontrolle.*

Nr.	Diätdauer Tage	Anfangsgewicht der Kaninchen g	Endgewicht der Kaninchen g	Knochen	Gewicht des Präparats mg	Initialer anorg. P mg	Abgegebener anorg. P mg
K 11	14	450	450	H	1440	0	0.44
				RU	1050	0	0.53
				RU	1150	0	0.47
K 3	30	300	210	H	900	0	0.31
				H	900	0	0.34
K 13	43	330	420	H	1300	0	0.29
				H	1330	0	0.31
				RU	1000	0	0.31
				RU	900	0	0.33
K 12	44	350	410	H	1350	0	0.31
				H	1350	0	0.34
K 4	57	520	480	RU	1700	0	0.29
				RU	1650	0	0.31
Durchschnittswert: Vorderbeine					**1232**	**0**	**0.35**

TABELLE 45. *Retention von anorganischem Phosphor in Kaninchenknochen.* (10 ccm physiologische Natriumchloridlösung) *D-Avitaminose.*

Nr.	Diätdauer Tage	Anfangsgewicht der Kaninchen g	Endgewicht der Kaninchen g	Knochen	Gewicht des Präparats mg	Initialer anorg. P mg	Abgegebener anorg. P mg
K 5	9	435	280	H	1315	0	0.34
				H	1335	0	0.38
K 6	24	395	300	H	1250	0	0.36
				RU	900	0	0.33
K 14	29	290	270	H	1000	0	0.40
				H	1100	0	0.47
K 15	29	320	300	H	1150	0	0.50
				H	1070	0	0.77
K 7	30	360	320	H	1120	0	0.24
				RU	820	0	0.38
K 10	30	330	340	H	1220	0	0.50
				RU	850	0	0.40
Durchschnittswert: Vorderbeine					**1094**	**0**	**0.42**

Gewebe darstellen, wurde in diesem Versuche die Herausdiffundierung nur dieser Fraktion beobachtet.

Die Tabellen 44 und 45 zeigen, dass die Präparate der Kontroll-Serie durchschnittlich eine Phosphormenge von 0.35 mg im Gegensatz zu derselben von 0.42 mg der »Rachitisversuche» abgegeben hatten, was auf eine Tendenz der Knochen im letzteren Falle Phosphor schwächer fixiert haben zu können deuten dürfte. Irgendwelche unmittelbare Schlüsse dürfte man jedoch kaum hieraus ziehen können, da die abgegebene Phosphormenge in der »Rachitisserie» in recht grossem Ausmasse variieren zu können scheint.

H. Besprechung der Versuchsergebnisse bei D-Hypervitaminose und bei D-Avitaminose.

Überblickt man die Ergebnisse der verschiedenen, im letzten Teile dieser Arbeit beschriebenen Versuche, so ergibt es sich, dass die Einwirkung des D-Vitamins auf den Calciumstoffwechsel in den Präparaten eine einheitliche Tendenz aufzuweisen scheint, wogegen der Effekt des Vitamins auf den entsprechenden Phosphormetabolismus nicht ebenso auffallend ist.

Die einleitenden Beobachtungen bei der D-Hypervitaminose zeigen, dass das Vitamin D einen stimulierenden Einfluss auf die Aufnahme von Calcium ins Knochengewebe hat. Wie kann man sich die Entstehung dieses Effekte vorstellen? Bei der Beurteilung dieser Frage muss in erster Reihe beachtet werden, dass das Inkubationsmilieu in den Kontrollversuchen und den Parallelserien genau gleich gewesen ist. Unter solchen Verhältnissen kann der Effekt des D-Vitamins auf keine andere Weise als vermittelst einer unmittelbaren Einwirkung auf im Knochengewebe selbst enthaltene Faktoren, welche auf irgend eine Weise im Mechanismus der Aufnahme von Calcium mit einbegriffen sind, entstanden sein. Den Versuchen mit normalem Knochengewebe nach zu urteilen, scheint diese Aufnahme mit einem aktiven Prozesse verglichen werden zu können, weshalb die Vermutung nahe liegen dürfte, dass der Effekt des D-Vitamins diesen Vorgang unmittelbar stimulieren könnte. Was den näheren Wirkungsmechanismus des Vitamins betrifft, so können verschiedene Möglichkeiten in Betracht kom-

men. In erster Reihe scheint man vielleicht die Möglichkeit in Erwägung nehmen zu können, dass das D-Vitamin einen Einfluss auf die im Knochengewebe herrschenden Permeabilitätsverhältnisse ausüben dürfte. Diese Annahme steht im Einklange mit der früher erwähnten Ansicht in einer u. a. von BOND, HARRIS und NICOLAYSEN vertretenen Studienrichtung, dass das D-Vitamin die Aufnahme von Calcium in verschiedene Gewebe befördere, da es deren Diffusionspermeabilität für diesen Stoff erhöhen solle.

Sollte man andererseits von der Annahme ausgehen, dass das Calcium bei der Aufnahme in Präparate wenigstens zum Teil von Proteinsubstanzen adsorbiert wird, so scheint es nicht ausgeschlossen zu sein, dass das D-Vitamin auf irgend einer Weise eine solche Bindung befördern könnte. Im Kenntnis des ungleich starken Vermögens verschiedener Proteinsubstanzen Calcium zu binden, ist es nicht undenkbar, dass dem D-Vitamin gleichzeitig eine Rolle bei der Bildung von einem eine besonders starke Calciumaffinität besitzenden Proteinkomplex zukommen könnte.

Ohne dass die Versuche eine unmittelbare Antwort auf diese verwirkelte Frage geben können, dürfte des weiteren die Möglichkeit in Erwägung gezogen werden, dass das D-Vitamin in irgendeinem nahen Zusammenhange mit den im Knochengewebe tätigen spezifischen osteogenetischen Prinzipien, welche in der letzten Zeit ein immer lebhafteres Interesse erweckt haben, stehen könne. Insbesondere da es bekannt ist, dass das dem Organismus zugeführte D-vitamin in fettreichen Geweben, unter welchen der Knochenmark eine hervorragende Rolle spielt, aufgespeichert wird, ist es nicht undenkbar, dass das Vitamin bei der Bildung und der eventuellen Aktivierung dergleichen lokalen osteogenetischen Prinzipien im Knochengewebe von unmittelbarer Bedeutung sein kann.

In diesem Zusammenhange wäre es angebracht noch eine von ENGSTRÖM und ORELL (1943) neulich gemachte, die Eigenschaften solcher die Knochenbildung hervorrufenden spezifischen Stoffe beleuchtende Beobachtung zu erwähnen. Bei subcutaner Implantation von vor dem Versuche, in einigen Fällen bis auf —190° herab, abgekühlter Knochenpräparate, konnten die genannten Forscher nach 4—6 Wochen Zeichen einer Neubildung von Knochen in der Umgebung des Implantats entdecken. Diese Beo-

bachtung ist auch insofern von Interesse, dass sie zeigt, dass das Knochenpräparat trots der Abkühlung Stoffe enthält, welche das subcutane Gewebe zur Knochenbildung anregen können. Das Überleben des Knochengewebes selbst scheint also keine notwendige Voraussetzung für die Erreichung dieses Effektes zu bilden.

Gelegentlich dieser Frage dürfte des weiteren erwähnt werden, dass auch NICOLAYSEN auf Grund seiner letzten Versuche (1943) von der Existenz eines »endogenen», die Aufnahme von Calcium bei jungen Ratten befördernden Faktors spricht. Die Abhängigkeit dieses Faktors vom Vitamin D ist daraus zu ersuchen, dass er keine Wirkung beim Fehlen des Vitamins zu haben scheint.

Aus meinen Versuchen geht hervor, dass das D-Vitamin, indem es das Vermögen des Knochengewebes Calcium aufzunehmen erhöht, gleichzeitig auch eine, wenn auch nicht so offenbare, Neigung einer stärkeren Fixierung dieser Substanz in den Präparaten hervorruft. Diese Wahrnehmung dürfte als ein weiteres Anzeichen dafür dienen, dass ein naher Zusammenhang zwischen diesen beiden Seiten des Calciummetabolismus im Knochengewebe besteht. Bei der Beurteilung der Frage, wie denn die stärkere Fixierung zu stande kommt, liegt die Vermutung nahe, dass das D-Vitamin auch hierbei auf zellulare und enzymatische Prozesse, welche auf irgend eine Weise die Fixierung von Calcium im Knochengewebe regulieren, einwirken könne. Man kann sich vielleicht vorstellen, dass dieselben Faktoren, welche die Aufnahme von Calcium ins Knochengewebe befördern, auch eine stärkende Wirkung auf die Fixierung dieser Substanz ausüben. Dem Umstande, dass das D-Vitamin den »Calciumshunger» des Knochengewebes erhöht, würde in diesem Falle eine doppelte Wirkung zu grunde liegen.

Im Zusammenhange mit der Literaturübersicht wurde die Beobachtung erwähnt, dass die Zuführung von D-Vitamin in allzu grossen Dosen eine Demineralisation vom Typus »ostitis fibrosa» mit sich ziehende Störungen im Stoffwechsel des Knochengewebes hervorrufen können. Es ist deshalb vorstellbar, dass eine solche Wirkung bei Versuchen in vitro in einer Verminderung der Aufnahme oder der Fixierung von Calcium im Knochengewebe Ausdruck finden können. Der Umstand, dass eine solche Wirkung des D-Vitamins bei meinen Versuchen nicht verspürt worden ist, dürfte vielleicht darauf zurückzuführen sein, dass die verabreichte Vita-

minmenge zu klein oder dass eher die Zuführungszeit allzu kurz gewesen ist um irgend welche Nebenwirkungen ausser dem positiven Effekt des D-Vitamins auf den Calciummetabolismus im Knochengewebe zum Vorschein kommen zu lassen. Die Frage, welcher Mechanismus der von einer Überdosierung von Vitamin D hervorgerufenen demineralisierenden Einwirkung auf das Skelett überhaupt zu grunde liege, ist nicht näher untersucht worden. Im Kenntnis der knochenauflösenden Wirkung gewisser Hormone, insbesondere desjenigen der Parathyreiodea, erscheint es jedoch nicht unmöglich, dass eine allzugrosse Überschreitung der optimalen Dosis D-Vitamins zu einer Überproduktion dieser Hormone führen könne.

Die Wahrnehmung, dass in meinen Versuchen das D-Vitamin keinen ebenso ausgeprägten Effekt auf den Phosphor- wie auf den Calciumssoffwechsel auszuüben scheint, dürfte wahrscheinlich nicht allzu kathegorisch gedeutet werden. Es ist ausserdem schwer, irgendwelche endgültige Schlussfolgerungen über diese Seite des Wirkungsmechanismus des D-Vitamins aus den Resultaten zu ziehen, weil die Versuche gezeigt haben, dass sowohl die Aufnahme, wie die Fixierung des organischen Phosphors, was deren Intensität betrifft, bei der Hypervitaminose sowohl in den Kontroll- wie in den Parellelserien zwischen recht bedeutenden Grenzen schwanken zu können scheint.

Eine Beobachtung von Cohn und Greenberg (1939), die im gewsisen Einklange mit meinen Ergebnissen steht, verdient in diesem Zusammenhange erwähnt zu werden. Die genannten Forscher konnten im Anschluss an ihre Versuche mit radioaktivem Phosphor keinen Effekt des D-Vitamins auf die Aufnahme von organisch gebundenem Phosphor im Knochengewebe von Ratten verspüren. Dagegen erwies das D-Vitamin sich in einigen Fällen die anorganische Phosphorfraktion erhöhen zu können. Die fraglichen Versuche führten zum folgenden Schlussatze: »The logical conclusion is that the vitamin acts to aid the conversion of organic to inorganic phosphorus, and that the transfer of phosphorus from blood to the organic fraction of bone is independent of vitamin D». Entsprechende Untersuchungen sind auch von Morgareidge und Manly (1939) ausgeführt worden, welche keinen Zusammenhang zwischen D-Vitamin und der Aufnahme von anorganischem radioaktiven Phosphor in Knochendiaphysen nachweisen konnten. Dage-

gen solle das Vitamin die Aufnahme von Phosphor in Metaphysen befördern.

Sollte man die Aufnahme von organischem Phosphor in einen Zusammenhang mit der Phosphatasetätigkeit in den Knochen-Präparaten stellen, so kann die ausgebliebene Wirkung des D-Vitamins auf diese Aufnahme in meinen Versuchen vielleicht als ein Ausdruck dafür, dass das D-Vitamin auf diese Enzymetätigkeit keinen Einfluss ausübe, gedeutet werden. Für die Richtigkeit einer solchen Annahme spricht zum Teil die Beobachtung, dass die Menge der finalen anorganischen Phosphorfraktion, welche wenigstens zum Teil durch die Einwirkung von Phosphatas auf das Natriumglycerophosphat in der Inkubationslösung entstehen dürfte, keinen auffallenden Unterschied in den Kontrollversuchen und den Parallelserien bei D-Hypervitaminose aufwies. Allerdings muss bei der Erörterung dieser Frage der Umstand beachtet werden, dass auch die finale anorganische Phosphorfraktion, was deren Grösse betrifft, recht ansehnlich bei den verschiedenen Versuchen schwankte, weshalb auch in diesem Falle keine endgültige Schlussfolgerung in die Rede kommen zu können scheint.

Bei den Versuchen trat in den Knochenpräparaten eine Neigung hervor organisches Phosphor bei gleichzeitigem Anwesen von Calcium in der Inkubationslösung bei D-Hypervitaminose stärker als in den Kontrollserien aufzunehmen. Es erscheint deshalb nicht ausgeschlossen, dass die stärkere Aufnahme von Calcium bei D-Hypervitaminose zu gleichzeitiger Herbeiführung einer entsprechenden Wirkung auf den Phosphormetabolismus beitragen dürfte. Diese Annahme steht im Einklange mit der früher erwähnten Beobachtung von FREUDENBERG und GYÖRGY, dass Calcium auf die Aufnahme von Phosphor in das Knochengewebe in vitro einen stimulierenden Einfluss ausübe, da dieselbe in direktem Verhältnis zur primären Anreicherung von Calcium stand. Es hält schwer schlussgiltig zu entscheiden, worauf dieser Effekt von Calcium auf die Aufnahme von organischem Phosphor zurückgeführt werden dürfte. Es ist jedoch nicht ausgeschlossen, dass das Calcium bei der Aufnahme in das Knochengewebe eine aktivierende Einwirkung auf die Phosphatasetätigkeit in demselben ausüben kann. Das Calcium dürfte somit dieselbe Wirkung haben, wie es bekanntlich mit dem Magnesiumion der Fall ist.

Als ein weiterer Beleg dafür, dass der Phosphorstoffwechsel im Organismus in einer nahen Abhängigkeit von der Anwesenheit von Calcium zu stehen scheint, dürfte in diesem Zusammenhange die Auffassung erwähnt werden, welche geltend gemacht hat (BAILEY sowie NEEDHAM 1942), dass das Myosin, dem in jüngster Zeit die Rolle eines Adenylpyrophosphorsäure spaltenden Enzyms zugeschrieben worden ist, das Vorhandensein von Calciumionen voraussetzt. Gemäss diesen Forschern soll die Erhöhung der Aufnahme von Calcium in das Muskelgewebe einer von den Effekten des D-Vitamins sein, welcher zu einer Aktivierung des Myosins führen und den Phosphormetabolismus auf diese Weise stimulieren dürfte.

Irgend welche die Einwirkung des D-Vitamins auf die Aufnahme von anorganischem Phosphor ins Knochengewebe beleuchtenden Versuche wurden in meiner Arbeit nicht veranstaltet. Die von v. KRAEMER und LANDTMAN (1940) ausgeführten Versuche deuten jedoch darauf hin, dass das D-Vitamin auf die Unfähigkeit des Knochengewebes diese Phosphorverbindung in vitro aufzunehmen keinen Einfluss ausüben zu können scheint.

Bei der Prüfung der bei D-Avitaminose erzielten Versuchsresultate ist das mangelhafte Vermögen der Rattenknochen Calcium aufzunehmen besonders auffallend. Dieser Effekt steht also im direkten Gegensatz zu den Ergebnissen der bei D-Hypervitaminose ausgeführten Versuche. Bei der Beurteilung dieser Wahrnehmung muss beachtet werden, dass auch die Präparate der Kontrollserie, in welchen die Versuchstiere 50 I. E. D-Vitamin täglich ausser der Diät Mc COLLUMS erhielten, eine gleiche, wenn auch nicht ebenso durchgängige Unfähigkeit Calcium aufzunehmen aufwiesen. Dass diese Vitaminmenge aber genügend gross war um Rachitiserscheinungen vorzubeugen, zeigte die Beobachtung, dass die Kontrolltiere weder klinische noch röntgenologische Anzeichen dieser Krankheit aufzuweisen hatten. Die mangelhafte Aufnahme von Calcium bei diesen Versuchen dürfte wahrscheinlich darauf zurückgeführt werden, dass, ausser dem Mangel an Vitamin D, die Zusammensetzung der Diät Störungen im Calciummetabolismus des Knochengewebes herbeiführen könne. Man kann sich auch vorstellen, dass das D-Vitamin allein die normale Fähigkeit des Knochens, Calcium während der in vielen Fällen langen Diätzeit aufzunehmen, völlig aufrecht zu erhalten nicht im Stande gewesen

ist. Das Sachverhältnis scheint jedoch in den Parallelserien bei Rachitis, in welchen die Knochen typische Symptome dieser Krankheit aufweisen, teilweise anders zu sein. In diesem Falle liegen wahrscheinlich die von dem Mangel an Vitamin D hervorgerufenen rachitischen Knochenveränderungen der gestörten Aufnahme von Calcium als Hauptanlass zu Grunde.

Welcher Mechanismus dürfte denn als Ursache der Unfähigkeit der Knochenpräparate Calcium in diesen Versuchen aufzunehmen angesehen werden können? Gewisse Tatsachen sprechen für das Vorhandensein verschiedener lokalen »Hemmungsmomente» als Ursache der rachitischen Knochenveränderungen. Man ist z. B., ziemlich allgemein der Meinung, dass Rachitis von einem verlangsamten Stoffwechsel und einer unvollständigen Oxydation der Zersetzungsprodukte, was einen wenn auch kompensierten acidotischen Zustand zur Folge hat, gekennzeichnet wird. (Pritchard 1922, Rabl 1923, Freudenberg und Welcker 1926, György 1928 u.a.). Die Wahrnehmung von Freudenberg und György (1921), dass gewisse saure Zersetzungsprodukte, hauptsächlich die von Eiweisstoffen, stark hemmend auf die Adsorption von Calcium im Knochengewebe in vitro wirken, dürfte als Stütze für die Annahme dienen, dass diese »Ketos bereitschaft» in der mangelhaften Aufnahme von Calcium ins Knochengewebe bei Rachitis einen Anteil habe. Von solchen Stoffen kamen u.a. Aminosäuren, Peptide und Ammoniumsälze in die Rede. Da gemäss diesen Forschern die primäre Anreicherung des Knochengewebes mit Calcium eine Voraussetzung für die weitere Mineralisierung des Skeletts ausmacht, wäre dadurch schon die erste Phase der Ossifikation gestört worden sein. Wenn von Substanzen, die eine hemmende Wirkung auf die Knochenbildung ausüben können, die Rede ist, dürfte noch erwähnt werden, dass Robison, Mc Leod und Rosenheim (1930) im Anschluss an ihre in vitro Versuche gezeigt haben, dass 4 mg % Magnesium in der Inkubationslösung die Calcifikation von Epiphysenknorpel inhibierten. Da rachitische Knochen bekanntlich einen erhöhten Magnesiumgehalt aufweisen, dürfte dieses als ein Teilfaktor beim Entstehen von Skelettveränderungen bei dieser Krankheit angesehen werden können.

Andererseits muss auch mit in Rechnung gezogen werden, dass die mangelhafte Aufnahme von Calcium ins Knochen-

gewebe bei Rachitis seinen Anlass auch in irgendeiner Form von Hypofunktion in der Wirksamkeit der unter normalen Verhältnissen den Ossifikationsprozess im Knochengewebe *stimulierenden* Faktoren finden könnte. PFAUNDLER, der als erster diese Annahme mehr eingehend ausgesprochen hat, giebt derselben den folgenden Ausdruck: ». . .Es ist vorstellbar, dass es sich um eine funktionelle Störung im Leben jener Zellen handelt, von denen aus ein aktives Prinzip auf die umliegenden Gewebsmassen umgestaltend einwirken soll.»

Bei der Besprechung dieses Problems kann erwähnt werden, dass einige schwedische Forscher in der allerletzten Zeit geltend gemacht haben, dass die formale Genese der Rachitis hinsichtlich der Skelettveränderungen darin bestehen würde, dass die Zellenteilung und Differenzierung mit abnormer Langsamkeit vor sich gehen sollte (FREUDENTHAL 1939, WILTON 1939, HÄGGQVIST, WILTON und ORELL 1944). Somit konnte WILTON in seinen Versuchen mit Dentingewebe nachweisen, dass die Demineralisierung bei Rachitis anfänglich die Wurzelzellen betrifft, was seine Erklärung in deren niedrigem Differenzierungsstadium finden dürfte. Als ein Beispiel dafür, dass eine Art von Hypofunktion im rachitischen Knochengewebe zu bestehen scheint, kann des weiteren hervorgehoben werden, dass NICOLAYSEN und NORDBØ (1943) bei ihren Versuchen, in denen sie die Citronensäure in rachitischem Knochengewebe bestimmten, eine auffallende Abnahme von diesem Stoffe im Vergleich mit normalen Verhältnissen beobachtet haben. Es dürfte jedoch schwer fallen zu entscheiden, ob diese Abnahme im Hervorrufen der rachitischen Knochenveränderungen einen Anteil haben würde.

Es scheint nicht möglich zu sein vollständige Klarheit darüber zu gewinnen, welche Faktoren der Störung in der Calciumaufnahme ins Knochengewebe bei Rachitis zu grunde liegen. Es ist jedoch nicht ausgeschlossen, dass diese Störung auch auf eine herabgesetzte Tätigkeit der spezifischen im Knochengewebe vorfindlichen »osteogenetischen Prinzipien» zurückgeführt werden könnte. Vielleicht wird die weitere Forschung auf diesem Gebiete ein klareres Licht auf dieses Problem werfen.

Meine entsprechende Versuche mit Kaninchenknochen zeigten, dass eine direkte Unfähigkeit Calcium aufzunehmen bei Rachitis

nicht vorzuliegen brauche. Auch in diesem Falle machte sich jedoch in der Kontrollserie bei der Zuführung von kleinen Mengen D-Vitamins ausser der rachitogenen Diät ein Bestreben zur stärkeren Adsorption fühlbar.

Dass auch rachitische Rattenknochen unter gewissen Bedingungen Calcium in vitro aufnehmen zu können scheinen, wird von den Versuchen SHIPLEY und ROBISONS, bei denen ausschliesslich rachitisches Knochenmaterial zur Anwendung kam, dargelegt, wobei jedoch das Anwesen von Phosphat in der Inkubationslösung eine Voraussetzung dieser Aufnahme war. Diese Beobachtungen stehen jedoch in keinem Widerspruch dazu, dass diese Calcifikation im Verhältnis zur Aufnahme von Calcium und Phosphor in normale Knochen schwächer sein kann. Sie schliessen auch nicht die Möglichkeit aus, dass beim Anwesen von nur Calcium in der Grundlösung die primäre Aufnahme von diesem Stoffe im Knochen-Gewebe bei Rachitis mangelhaft sein kann.

Die Beobachtung, dass nicht nur die Adsorption, sondern auch die Fixierung von Calcium im Knochengewebe bei D-Avitaminose geschwächt war, kann als ein weiteres Zeichen des engen Verhältnisses, das zwischen diesen beiden Vorgängen zu herrschen scheint, dienen. Die Annahme dürfte deshalb nahe liegen, dass die mangelhafte Mineralisierung des Skeletts bei Rachitis zum Teil ein Ergebnis des Zusammenwirkens dieser beiden Störungen des Calciumstoffwechsels sei. Man könnte sich vielleicht vorstellen, dass dasselbe Ursachsmoment diesem Doppeleffekt zu grunde liegt. Eine Stütze findet diese Annahme in einer Beobachtung von ULLRICH (1929), dass gleichartige Zersetzungsprodukte, die gemäss den Versuchen von FREUDENBERG und GYÖRGY eine hemmende Wirkung auf die Aufnahme von Calcium ins Knochengewebe ausübten, in vitro auch eine erhöhte Mobilisierung von Calcium aus der Knochensubstanz verursachten. Andererseits kann man auch nicht die Möglichkeit verneinen, dass auch ausser einem solchen Mechanismus ein Mangel an den die Fixierung von Calcium *stimulierenden* Faktoren zu einer erhöhten Auswanderung dieses Stoffes aus den Präparaten der Rachitisversuche führen könnte.

Hinsichtlich der Aufnahme von organischem Phosphor ins Knochengewebe bei D-Avitaminose konnte keine offenbare Abweichung von den normalen Verhältnissen verspürt werden. Im Lichte

der harmonierenden Ergebnisse der Versuche bei D-Hypervitaminose kommt es somit vor, als ob das D-Vitamin — im Ramen dieser in vitro-Experimente — keinen primären Einfluss auf den Phosphor-Metabolismus im Knochengewebe ausüben würde. Es ist jedoch wahrscheinlich, dass man in Anbetracht des recht geringen Materials und insbesondere im Kenntnis der verhältnismässig grossen Schwankungen, denen die Aufnahme von Phosphor in den früheren Versuchen unterworfen war, keine endgültige Schlussfolgerung hieraus ziehen dürfte. Stellt man jedoch, wie in der obigen Erörterung angenommen, die isolierte Aufnahme von organischem Phosphor in den Knochenpräparaten in einen Zusammenhang mit der Phosphatas wirksamkeit in denselben, so deuten die Versuche darauf hin, dass auch der Mangel an Vitamin D, wenigstens in keinem nennenswerten Masse, die Fähigkeit dieser Enzymen Phosphor primär zu adsorbieren nicht beeinflussen würde.

Auf welche Weise muss also die ausgebliebene Mineralisierung des Knochengewebes bei Rachitis, was den Phosphor betrifft, ihre Erklärung finden? Da wie bekannt die Phosphatas menge im Knochengewebe bei Rachitis meistens erhöht ist, taucht ein berechtigter Zweifel auf, ob diesen Enzymen eine führende Rolle eines die Aufnahme von Phosphor in die Knochen befördernden »lokalen Momentes» zugeschrieben werden kann. Dieser Gegensatz wird durch eine ziemlich allgemeine Annahme erklärt, nach welcher das rachitische Milieu aus dem einen oder anderen Grunde die Phosphatasetätigkeit hemmend beeinflussen sollte. Der niedrige anorganische Blutphosphor bei Rachitis ist als ein Ausdruck dafür, dass die hydrolysierende Wirkung der Phosphatase gestört ist, angeführt worden. Da, wie von Robison dargelegt, das Wirksamkeitsoptimum der Knochenphosphatase im alkalischem Gebiete liegt, dürfte vielleicht die Gewebsacidose bei Rachitis eine gewisse Rolle eines den Phosphatas mechanismus hemmenden Faktors spielen.

Andererseits scheint auch die Hypothese annehmbar zu sein, dass die bei Rachitis im Knochengewebe verminderte Phosphormenge auf irgendeine Weise die Folge einer mangelhaften Aufnahme von Calcium sein könne. Diese Annahme erscheint im Lichte der Untersuchungen von Freudenberg und György als besonders berechtigt, da diese Forscher, wie oben erwähnt, die primäre Auf-

nahme von Calcium ins Knochengewebe als einen Determinant für die Aufnahme von Phosphor und die weitere Ossifikation ansehen. Wird, gemäss diesen Forschern, die einleitende Phase des Verknöcherungsprozesses gestört, so soll alsdann die Anwesenheit von Phosphor zu keiner Fällung von Knochensalzen führen.

Gelegentlich der Abhängigkeit des Phosphors von dem Vorhandensein von Calcium im Knochengewebe, verdient eine neulich von MONETTI (1942) gemachte Beobachtung erwähnt zu werden, nach welcher die Pyrophosphorsäurefraktion im rachitischen Knochengewebe im Gegensatz zum organischen und totalsäurelöslichen Phosphor erhöht sein wäre. Im Lichte des obenerwähnten Erweises von BAILEY und NEEDHAM, dass die Spaltung von Pyrophosphat die Anwesenheit von Calcium voraussetzt, dürfte dieser erhöhte Pyrophosphatgehalt vielleicht in irgend einem Zusammenhange mit der im rachitischen Knochengewebe verminderten Calciummenge stehen.

Was die Retention von Phosphor im Knochengewebe bei Rachitis betrifft, so sind meine Versuchsergebnisse etwas widersprechend. Während bei den Versuchen mit Kaninchenknochen eine Neigung zur erhöhten Abgabe von Phosphor in der Rachitisserie verspürt werden konnte, war irgend ein entsprechender Effekt bei den gleichen Versuchen mit Rattenknochen nicht zu beobachten. Eine direkte Stellungsnahme dieser Seite des Phosphormetabolismus im Knochengewebe bei Rachitis gegenüber dürfte deshalb wohl kaum in die Rede kommen können.

V. Zusammenfassung.

Der Zweck der vorliegenden Arbeit war in erster Reihe einen Einblick in den Calcium und Phosphorstoffwechsel im Knochengewebe vermittelst in vitro und auf rein chemischen Wege ausgeführter Versuche zu erlangen. In dem letzten Abschnitte der Arbeit wurde die Aufmerksamkeit den mit derselben Methodik geführten vergleichenden Studien der von Vitamin D auf diesen Stoffwechsel ausgeübten Wirkung gewidmet, wobei das Problem von zwei Seiten beleuchtet wurde. Anfänglich wurde der Calcium- und Phosphormetabolismus untersucht, nachdem den Versuchstieren grosse Mengen D-Vitamin verabreicht wurden. Alsdann wurde der entsprechende Stoffwechsel bei D-Avitaminose studiert. Bei diesen Untersuchungen dienten als Versuchsobjekte mit der rachitogenen Kost Mc Collums aufgezogene Tiere.

Das Versuchsmaterial umfasste in allem 536 Vorder- und Hinterbeinspreparate von insgesamt 115 Versuchstieren, unter denen sich 67 Ratten, 32 Meerschweinchen, 15 Kaninchen und 1 Kalbembryo befanden.

Die Versuche führten zu den folgenden Ergebnissen:

A. *Normale Knochen.* Normaler Knochen besitzt die Fähigkeit aus ihn umspülender, Calcium enthaltenden Inkubationslösung diesen Stoff aufzunehmen. Als Grundlösungen wurde bei den Versuchen die Basallösung Robisons, physiologische Natriumchlorid-Lösung, sowie in einer Serie destilliertes Wasser angewandt. Der initiale Calciumgehalt der Versuchslösung entsprach im grossen ganzen dem normalen Calciumgehalt im Serum.

Bei den entsprechenden, die Aufnahme von Phosphor ins Knochengewebe beleuchtenden Untersuchungen wurde anfänglich

anorganischer Phosphor in der Form von sekundärem Natrium-Phosphat als Substrat angewandt. Es erwies sich jedoch, dass die Knochen Phosphor in dieser Form aufzunehmen nicht fähig waren. Bei diesen Versuchen enthielten die Inkubationslösungen bis auf 50 mg % anorganischen Phosphors.

Lag dagegen der Phosphor in der Form von organischem Phosphat vor — bei diesen Versuchen kam Na-Glycerophosphat zur Anwendung — so fand eine offenbare Aufnahme dieses Stoffes in die Präparate statt. Bei einer anfänglichen organischen Phosphorkonzentration von 18 mg % in der Inkubationslösung konnte bei Versuchen mit Meerschweinchenknochen eine Aufnahme von bis etwa 20 % der gesamten initialen säurelöslichen Phosphorfraktion beobachtet werden.

Die Versuche zeigen, dass Calcium und Phosphor im Knochen-Gewebe nicht stabil fixiert zu sein scheinen. Wurden nämlich die Knochenpräparate in calcium- und phosphorfreien Grundlösungen inkubiert, so erfolgte eine deutlich nachweisbare Auswanderung dieser Stoffe aus den Knochen.

Von den verschiedenen Teilen eines und desselben Knochens adsorbierte der Epiphysenknorpel bedeutend mehr Calcium und Phosphor als die Knochendiaphyse. Ausserdem wurden in dem ersteren eine grössere Fähigkeit diese Stoffe zu fixieren als in der letzteren beobachtet.

Ein Zusatz von Formaldehyd (3.5 %) zu der Inkubationslösung übte eine lähmende Wirkung auf die Aufnahme von Calcium und Phosphor in die Präparate aus. In diesem Falle wurde keine Adsorption, sondern eher eine Abgabe dieser Stoffe aus den Präparaten nachgewiesen. Ausserdem verminderte der Formaldehyd auch die Fähigkeit des Knochens Calcium und Phosphor in einer Basallösung zu fixieren, welche Wirkung sich dadurch äusserte, dass diese Stoffe beim Vorhandensein des genannten Agens aus den Knochen stärker als unter normalen Verhältnissen »leckten».

Der Zusatz von Toluol (2 %) in eine Phosphor enthaltende Inkubationslösung schwächte die Aufnahme dieses Stoffes in die Präparate. Die Wirkung war jedoch nicht ebenso prägnant wie im vorherigen Falle.

B. 1. *D-Hypervitaminose.* Nach Zuführung von Vigantol — 12000—36000 I. E. Vitamin D pro die — an die Versuchstiere,

wiesen die Knochen ein deutlich erhöhtes Vermögen Calcium zu adsorbieren auf. Die Knochenpräparate solcher Tiere nahmen durchschnittlich etwa 2—3 Mal mehr Calcium auf als die Knochen der Kontrollserien, in welchen den Versuchstieren entsprechende Volummengen reines Sesamöls verabreicht wurden.

Neben dieser Wirkung wurde in den Knochen eine schwache Neigung Calcium bei D-Hypervitaminose stärker zu fixieren verspürt.

Hinsichtlich der isolierten Aufnahme von Phosphor konnte irgend eine offenbare Wirkung des D-Vitamins nicht verspürt werden. Eine Neigung zur stärkeren Adsorption von Phosphor bei D-Hypervitaminose machte sich dagegen spürbar wenn auch Calcium in der Inkubationslösung vorhanden war.

Auch auf die Fixierung von Phosphor in Knochen schien das D-Vitamin keine nennenswerte Wirkung auszuüben.

Die Zuführung von Vitamin D erhöhte die Calciummenge im Serum, sowie auch die gesamte säurelösliche- und die anorganische Phosphorfraktion im Vollblut.

2. *D-Avitaminose.* Bei der Inkubation von ausschliesslich mit der rachitogenen Kost Mc Collums ohne Zusatz von Vitamin D aufgezogenen Ratten entstammendem Knochenmaterial erwies sich die Fähigkeit der Präparate Calcium aufzunehmen als stark vermindert. Anstatt einer Aufnahme fand in diesem Falle eine Abgabe von Calcium aus den Präparaten statt. Auch in den Kontrollversuchen, in denen die Ratten ausser dieser Kost 50 I. E. D-Vitamins täglich erhielten, wurde eine ähnliche, wenn auch nicht ebenso durchgehende Störung in der Calciumadsorption verspürt.

Bei den entsprechenden Versuchen mit Kaninchenknochen wurde sowohl in der Kontroll- wie in der Rachitisserie der meisten Versuche eine Adsorption von Calcium wahrgenommen. Diese war jedoch im Durchschnitt stärker in der Kontrollserie bei der Zuführung von Vitamin D (300 I. E. täglich).

Was die Fixierung von Calcium betrifft, so erwies sich dieselbe bei Versuchen mit Rattenknochen als etwas schwächer in den Rachitisserien denn bei den Kontrollversuchen.

Ebenso wie es bei der D-Hypervitaminose der Fall war, scheint auch anlässlich der Aufnahme von Phosphor in rachitische Rattenknochen kein schlussgültiger Urteil gefällt werden zu

können. Auch hatte die entsprechende Fixierung von Phosphor keine nennenswerten Unterschiede in den Kontroll- und Rachitisserien aufzuweisen. Bei Versuchen mit Kaninchenknochen machte sich dagegen bei den Präparaten der Rachitisserie eine Neigung spürbar diesen Stoff etwas schwächer als in der Kontrollserie fixieren zu können.

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